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Primary cultures of stromal-vascular (S-V) cells from adipose tissue were used to investigate the regulation of preadipocyte development. Differentiation of S-V cells was found to be under hormonal control. Insulin and glucocorticoids are essential for S-V cell differentiation in culture.

S-V cells from both newborn and mature pig adipose tissue and sera from both ages were used to examine the effect of age on preadipocyte development. S-V cells from newborn pigs replicated faster and appeared more responsive to serum borne factors influencing S-V cell growth and development in culture. Serum source (newborn vs mature) did not affect differentiation of S-V cells from newborn or mature pig adipose tissue.

When sera from fed or fasted pigs were used to culture newborn pig S-V cells, fasted pig sera stimulated greater differentiation and decreased cell replication as indicated by DNA content of rat S-V cell culture.

Lean pig serum compared to obese pig serum, increased differentiation activity in culture of S-V cells an effect which may be influenced by sex.

When sera from rat and pig were subjected to gel filtration fractionation on Sephacryl S-200 column, the elution profiles of both sera were similar. Rat serum contained six additional peaks (280 nm) not present in pig serum. Rat serum fraction two (apparent molecular size 67-150 kD) promoted greater differentiation of S-V cells than other rat serum fractions or pig serum fraction two. Fraction three (apparent molecular size 17-43 kD) of both sera inhibited differentiation and lipid filling in cultures of S-V cells but only rat fraction three promoted cell proliferation.

Rat and pig S-V cells have different morphology when differentiated. Differentiated rat S-V cells appeared as individual cells when cultured in serum free or serum supplemented medium while differentiated pig S-V cells appeared as individual cells in serum free medium and as a tight cluster of cells in serum supplemented medium. Both cells responded differently to sera obtained from pigs of differing ages and development of rat S-V cells was influenced by anatomic site.

# Regulation of Adipose Stromal-Vascular Cell Differentiation in Culture

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# Chapter 1

## INTRODUCTION

Increased muscle mass in animals signifies efficient animal and meat production in livestock and meat industry whereas increase in fat deposition connotes inefficiency. Some 3.63 billion kg of excess animal fat is produced yearly in the United States, and excessive fat in meat has become a major problem for the animal industry. Consumers are avoiding the consumption of excessive fat in meat because of the link between saturated fat of animal origin and coronary heart disease. Health consciousness of consumers has created an economic need for animal producers to maximize muscle production at the expense of other tissues. Scientists are involved in numerous research projects to help the meat industry respond to this new demand for high-quality lean meat.

Growth manipulation of agriculturally important animals can be enhanced by understanding the cellular and molecular bases of growth regulation of such important tissues as skeletal muscle, adipose tissue and bone. Particular emphasis has been put on increasing muscle accretion and decreasing fat deposition, but the nature of adipocyte development is not completely understood. Since growth and development of adipocytes and their aggregation to adipose tissue comprise an important phase in growth and development of animals, knowledge of conditions and factors that promote adipose tissue development would aid in devising methods to reduce body fat accretion and to enhance the production efficiency of meat producing animals. Therefore, it is pertinent to conduct further investigation to increase our understanding of adipocyte growth and development.

One of the methods employed in the study of adipocyte development is cell culture which allows investigators to monitor adipocyte development in a controlled environment. There are two different cell culture systems available: first, clonal cell lines, adipose-like cells grown in an artificial environment; and second, primary culture, stromal-vascular (S-V) cells obtained from adipose tissue samples and grown outside the animal for the first time. Each culture system has its advantages and disadvantages but both methods appear to be valid model systems to study the process of adipocyte differentiation at the cellular and molecular levels.

Using cell culture systems several hormones and growth factors have been shown to influence preadipocyte proliferation and differentiation in different species. However, suitable serum free media for cultures of stromal-vascular cells from meat producing animals have not been defined. Primary culture of pig stromal-vascular cells has largely been conducted in serum supplemented media. Serum contains many components which can confound the results of hormonal and/or growth factor treatments and since hormonal requirements have not been established to study differentiation of S-V cells from meat producing animals, a portion of this study was undertaken to investigate the effect of such hormones as insulin, glucocorticoid and triiodothyronine on the differentiation of porcine S-V cells in serum free culture. Investigation of such hormonal influences on the differentiation of preadipocytes in porcine S-V culture was needed in order to attempt to establish optimal chemically defined media to be used to study porcine preadipocyte differentiation. A second facet of the study determined variations in the ability of S-V cells obtained from pigs of different ages to differentiate, and the ability of sera obtained from pigs of

different ages to promote differentiation in culture. A third part of the study focused on the ability of sera from pigs of different sexes, genetic backgrounds and differing nutritional treatment as well as from different species to promote differentiation of cultured S-V cells obtained from rats or newborn pigs. These experiments were required in order to determine whether serum borne factors are responsible for differences in cellularity of young and mature pig adipose tissue and whether serum factors have a significant influence on the development of adipose tissue of different strains of pigs.

Although some factors promoting growth and development of preadipocytes in sera of other species have been identified, data suggest the possible existence of other yet unidentified factors. A final stage of this work included the fractionation of pig and rat serum by molecular weight and comparison of the influence of different serum fractions on growth and development of porcine S-V cells using rat serum fractions as references since some fractions in rat serum influencing preadipocyte differentiation and adipose conversion have been identified.

Utilization of these various approaches will help to elucidate factors and mechanisms controlling adipocyte development. Ultimately, the identification of hormonal, serum factor(s) and physiological control of preadipocyte growth should increase the potential for developing a physiological approach for manipulation of adipose tissue accretion in meat producing animals. We should therefore be able to increase production efficiency.

# LITERATURE REVIEW

**Adipose Tissue Accretion.** Adipose tissue is described as a specialized type of connective tissue made up primarily of adipocytes encircled by a collagen matrix; it is vascular and innervated and contains other cells characteristic of connective tissue (Slavin, 1985). Investigation of the order of growth at the cellular level shows three distinct phases (Winick and Noble, 1965): Cellular growth is first marked by proportional increases in cell size, protein and DNA content. This phase represents hyperplasia, in which there is an increase in cell number while individual cell size does not change. This stage of cell growth is followed by a transition phase which is characterized by concurrent hyperplasia and hypertrophy or increase in cell size; at this stage the rate of net DNA synthesis decreases while cell size and protein content continue to increase. Following this, further growth is by means of cell hypertrophy, with no further increase in DNA synthesis.

Adipose tissue mass may be controlled by both hyperplasia and hypertrophy (Hirsch et al., 1989). Overstimulation of these cellular processes ultimately results in obesity. The respective contribution of cell size and number to adipose cellularity can be determined during a particular stage of growth. Measurement of cell number in some breeds of pig during growth showed that cells continued to increase in number until 5 or 6 weeks of age (Gurr et al., 1977), indicating a hyperplastic period of adipose tissue growth exists. Severe feed restriction to suckling pigs did not change the number of adipocytes in the subcutaneous fat depot (Lee et al.,

1973 a,b). Since cell number in undernourished pigs did not differ from fully fed pigs as a result of suboptimal nutritional status, these results indicate that in the fetal or early postnatal period of growth adipocyte precursor cells proliferate (hyperplasia), and this establishes a precursor pool of cells that increases in size (hypertrophy). This however, does not mean that preadipocyte replication ceases. Preadipocytes in postnatal pig adipose tissue stromal fraction can proliferate and differentiate in culture (Novakofski, 1981).

Cellular differentiation and hypertrophy appear to occur more readily than replication in adipose tissue of postnatal pigs during growth (Anderson and Kauffman, 1973; Mersmann et al., 1975; Hood and Allen, 1977). The same phenomenon has been observed in the human (Naslun et al., 1988). Cell size distribution in meat producing animals has been reported (Anderson and Kauffman, 1973; Mersmann et al., 1975; Hood and Allen, 1977). Increasing adipose mass is associated with a biphasic distribution of cell sizes indicating that upon reaching a critical size adipocytes replenish the adipocyte pool with new cells (Allen, 1976; Hood, 1977; 1982). It is not, however, known whether the new cells come from division or delipidation of the existing mature adipocytes or from filling of precursor cells.

Methodology such as electronic particle counting (Coulter Counter) and microscopy are not adequate in enumerating cell number since adipocytes have to contain a certain amount of lipid before they can be counted. Therefore, data concerning hyperplasia are vague. Until there is a definitive marker to identify the adipocyte during early stages of differentiation, the contribution of hyperplasia in

increasing adiposity will remain unclear.

**Morphology of Adipose Tissue Development.** Histological features of white adipose tissue (WAT) are reviewed and discussed elsewhere (Slavin, 1985). Fixation and staining of adipose tissue shows primarily the shape of adipocytes as being spherical or oval. Adipocyte profiles may be distorted by solvent during lipid extraction in the processing procedure (Slavin, 1985). Lipid staining with oil red O shows that adipocytes contain a large centrally located lipid droplet. Other cell types such as macrophages, fibroblasts, mast cells and leukocytes are notable in WAT. Adipocytes in WAT are arranged into lobules which are braced by loose connective tissue stroma (septa), and individual adipocytes are supported by a network of reticulum and collagen fibers that are extended from interlobular septae. Based on histological and enzymological studies, several cell types have been identified as precursors to adipocytes. Cells suggested as adipocyte precursors include endothelial cells (Cameron and Smith, 1964; Desnoyers and Vodovar, 1977), perivascular reticulum cells (Wasserman, 1965; Simon, 1965; Sheldon, 1969; Hermans, 1973) and fibroblast or fibroblast-like cells (Napolitano, 1963). The results of studies on the origin of adipocytes have been equivocal. Histological and enzymological methods to document the developmental sequence of the adipocyte have been ineffective (Van, 1985).

**Adipose Precursor Cells in Culture.** Although the embryonic origin of adipocyte is doubtful, in vivo injection of a $^{3}$H-thymidine shows that newly synthesized

adipocytes come from primordial cells in the stromal fraction of adipose tissue (Hollenberg and Vost, 1968; Greenwood and Hirsch, 1974; Gaben-Cogneville and Swierczewski, 1979). Culture of adipose tissue S-V cells obtained from human (Adebonojo, 1975; Van et al., 1976; Hauner et al., 1989), rat (Van and Roncari, 1978; Bjorntorp et al., 1978, 1980), sheep (Broad and Ham, 1983) or pig (Hausman et al., 1984; Ramsay et al., 1989a,b) under suitable conditions results in morphological changes and increased expression of lipogenic enzymes typical of adipocytes and identified with accumulation of lipids. The growth of S-V cells in culture allows the evaluation of hormones and growth factors influencing growth and development of preadipocytes. Similarly, cell lines have been established to study the physiology and development of adipocytes. Adipocyte-like cell lines, 3T3 fibroblasts developed from the Swiss mouse embryo (Green and Kehinde, 1974; 1975) have been given a great deal of attention over the decade. The ob17 cells and subclones originating from epididymal fat pads of genetically obese ob/ob mice (Negrel et al., 1978) have also been extensively studied. When cultured in suitable media cell lines assume the phenotype and biochemical characteristics of mature adipocytes. Lipogenic enzymes such as lipoprotein lipase (Wise and Green, 1978; Spooner et al., 1979) and sn-glycerol-3-phosphate dehydrogenase (Grimaldi et al., 1978; Pairault and Green, 1979) are expressed and differentiation-dependent changes in gene expression are observed during adipose conversion of 3T3 cells (Spiegelman et al., 1983; Bernlohr et al., 1985; Cook et al., 1985; Philips et al., 1986).

**Serum-free Medium for Culture of S-V cells.** Serum contains many components of which some are poorly characterized or have not been identified. Use of serum in cell culture has inherent disadvantages in the study of hormones and factors regulating adipocyte development. The presence in serum of specific growth factors and several undefined factors can confound the results of studies designed to evaluate the roles of these and other serum components that influence growth and development of adipose precursor cells. Therefore, culture conditions are needed to provide a defined environment for undertaking comprehensive studies of the regulation of adipocyte development and the physiology of lipid accumulation of normal cells. Recently, developments have been made in primary culture techniques to permit the study of adipose S-V cells in culture which make it possible to identify factors controlling proliferation and differentiation of S-V cells from rat adipose tissue (Deslex et al., 1987a; Serrero and Mills, 1987), sheep adipose tissue (Broad and Ham, 1983), rabbit adipose tissue (Reyne et al., 1989) and human adipose tissue (Hauner et al., 1989; Deslex et al., 1987b). Serum free system similar to that described by Reyne et al., (1989) has been used successfully for the culture of porcine adipose tissue S-V cells (Suryawan, 1990). With optimization, the serum free medium (Suryawan, 1990) provides us with a suitable system to examine the development of porcine adipose precursor cells maintained in a defined culture environment.

**Nutritional Manipulation Effect on Adipocyte Development.** Varying caloric intake of the young rats during the suckling period by manipulating litter size early

in life showed that rats nourished in small litters had more fat cells in the epididymal fat pad than those nourished in large litters (Knittle and Hirsch, 1968). Restriction of feed intake to pigs after hyperplastic growth (6 to 40 weeks of age) induced weight loss but did not reduce the fat cell number (Gurr et al., 1977) Upon removal of feed restriction adipocyte number appeared to increase (Gurr et al., 1977). Undernutrition of rats followed by rehabilitation resulted in a rapid deposition of body fat (Harris, 1980). Chronic undernutrition impaired fat cell replication and rehabilitation from undernutrition caused increase cell replication in the subcutaneous fat depots (Kirtland and Harris, 1980). These results indicate that upon applying nutrient restriction, mature fat cells deplete their lipid contents ultimately causing weight loss, but the cells remained committed and became filled when there was abundant food supply. The source of the new cells contributing to the increase in cell number is not known. New cell may arise from precursor cells or from the dedifferentiation of mature cells. Using "ceiling culture", Sugihara et al.(1986; 1987) have shown that mature adipocytes can dedifferentiate and divide after depleting their fat stores. In contrast to the results in pigs and rats, dietary restriction over a period of time in overweight women caused weight loss and a reduction in both fat cell size and number (Sjostrom and William-Olsson, 1981). A number of studies suggest that diet composition can influence adipose cellularity. Feeding rats a diet containing elevated saturated fat as opposed to unsaturated fat resulted in an increase in fat cell number without any significant weight gain (Lemonnier and Alexiu, 1974). An increase in adipocyte number can result from overfeeding a palatable high fat diet to rats (Faust et al., 1978; Klyde

and Hirsch, 1979). Feeding of high fat diets to lean and obese pigs resulted in more carcass adipose tissue deposition in both strains of pigs (Mersmann et al., 1984). Although cellularity data associated with the increased adipose tissue deposition was not provided, at least two phenomena are clearly possible: increase in size of existing differentiated adipocytes and increase in adipocyte number resulting from proliferation and differentiation of primary preadipocytes. Overfeeding of rats resulted in changes in sera to support differentiation and proliferation of adipocyte precursor cells in culture (Jewell et al., 1988). Jewell et al. (1988) found that sera from overfed (tube-fed) rats were able to decrease adipocyte proliferation more than sera from ad libitum fed rats, and sera from tube-fed rats were able to enhance preadipocyte differentiation better than sera from ad libitum fed rats. Bjorntorp et al. (1985), however, found that dietary status did not change the ability of plasma to promote proliferation and increase the activity of glycerol-3-phosphate dehydrogenase when sera were added to cultured cells around the time of confluence, but it did change the rate at which differentiated cells filled with lipids. The effect of dietary status on the ability of plasma to affect the rate of lipid-filling of cultured preadipocyte was attributed to the variations in their very low density lipoprotein contents. From all of the above, it is clear that nutritional manipulation can be useful to study adipocyte development.

**Steroid Hormone Effect on Adipocyte Development.** Administration of estrogen or synthetic glucocorticoid, dexamethasone in rats decreased adipocyte lipid content while progesterone injection elevated lipid deposition in adipocytes (Krotkiewski and

Bjorntorp, 1975; Wade and Gray, 1979; Steingrimsdottir et al., 1980). In cultures of adipocyte-like cell lines and adipose S-V cells, steroid hormones such as glucocorticoids promote adipocyte differentiation. The stimulation of differentiation by steroid hormone has been demonstrated in rat adipose precursor cells incubated with progesterone (Xu and Bjorntorp, 1987). After treating sera by heat to remove any heat sensitive factors (Hauner and Loffler, 1986) or performing immunoaffinity purification with growth hormone antibodies (Nixon and Green, 1984) to remove growth hormone, sera retained part of the adipogenic activity which was attributable to glucocorticoids (Schiwek and Loffler, 1987). Cortisol and corticosterone are steroid hormones promoting most actively the adipose conversion in clonal cell lines (Feick and Loffler, 1986). Mineralocorticoids have weaker glucocorticoid activity and can promote adipose conversion of preadipocytes only when pharmacological concentrations are used, while estrogens possess no adipogenic activity (Feick and Loffler, 1986). The effects of glucocorticoids have also been demonstrated in cultures of S-V cells obtained from rats (Wiederer and Loffler, 1987; Gaben-Cogneville et al., 1990), humans (Hauner et al., 1987;1989) and pigs (Ramsay et al., 1989a). Recently, Xu and Bjorntorp (1990) found that the effect of dexamethasone on rat adipose precursor cell growth depends on the type of medium, time period of exposure and the presence of insulin. Effect of glucocorticoids on adipose conversion of cells was found to be augmented only in the presence of insulin. Combination of insulin and glucocorticoids has been shown to promote adipogenic conversion of cultured TA1 cells (Chapman et al., 1984). Dexamethasone in combination with insulin caused the accumulation of mRNA that

are induced during adipocyte development of TA1 cells (Chapman et al., 1985). It has been suggested that glucocorticoid effect on the expression of lipogenic enzymes is similar to that of insulin (Gaben-Cogneville et al., 1990). However, recent studies (Moustaid et al., 1990) have shown that dexamethasone decreases glycerol-3-phosphate dehydrogenase mRNA level, a late marker of preadipocyte differentiation during adipose conversion of 3T3-F442A cells. The results on the effect of dexamethasone on gene expression in adipose cell lines during differentiation are contradictory. It is possible that the different cell lines used in the above mentioned studies have undergone alterations involving genetic changes which allow them to respond differently to the treatment.

**Insulin Effect on Adipocyte Development.** One of the most important hormones regulating adipose tissue metabolism is insulin (Mersmann, 1979). Physiological concentrations of insulin enhanced lipogenic capacity and promoted almost complete differentiation in primary culture of rat preadipocytes (Gaben-Cogneville et al., 1983). Other researchers (Wiederer and Loffler, 1987; Hausman and Jewell, 1988) have also demonstrated the influence of insulin on the differentiation of S-V cells from rat adipose tissue. Hausman and Jewell (1988) used fetal or postnatal pig sera supplemented with various concentrations of insulin to evaluate the influence of insulin on cell differentiation. Fetal pig serum is inherently low in insulin. Addition of $10^{-9}$M insulin increased the number of esterase positive cells, and cells showed more lipoprotein lipase activity (Hausman and Jewell, 1988), indicating that insulin promoted differentiation. Insulin alone did not promote

differentiation in rat primary culture (Li et al., 1989). The disparity between this result (Li et al., 1989) and the report of Gaben-Cogneville et al., (1983) was attributed to the differences in age of rats used. Addition of insulin to pig S-V cell cultures did not affect glycerol-3-phosphate dehydrogenase activity (Hentges and Hausman, 1989), indicating that S-V cells from different species respond differently to insulin. Influence of insulin on adipose conversion of some adipocyte-like cell lines also has been demonstrated. Within physiological concentrations, insulin enhanced the adipose conversion of ob17 cells (Grimaldi et al., 1983a,b; Amri et al., 1984). Insulin is however required at pharmacological levels in 3T3 cells (Green and Kehinde, 1975; Spooner et al., 1979; Rosen et al., 1979). Other cell lines whose differentiation is influenced by insulin include CHEF/18 cells (Sager and Kovac, 1982; Harrison et al., 1985). Insulin increases activity of lipogenic enzymes such as lipoprotein lipase, glycerol-3-phosphate dehydrogenase, and acetyl CoA carboxylase and promotes the accumulation of the mRNA for glycerol-3-phosphate dehydrogenase (Alexander et al., 1985; Dani et al., 1986; Moustaid et al., 1990).

Replication of 3T3-L1 preadipocyte cell line (Gamou and Shimizu, 1986) and porcine S-V cells (Duliere et al., 1991) is stimulated by insulin. Insulin at a pharmacological level could bind to the high-affinity receptor of insulin-like growth factor-1 (IGF-1) (Hausman et al., 1989). Insulin may stimulate the secretion of somatomedin-like peptide in smooth muscle (Clemmons, 1985), which in turn can stimulate DNA synthesis (Clemmons and Van Wyk, 1985). Stimulation of replication by insulin can be as a result of either or both of these possibilities.

**Growth Hormone Effect on Adipocyte Development.** Growth hormone increases muscle accretion and decreases fat deposition (Etherton, 1989). The effect of growth hormone on adiposity has been attributed to its antagonistic action on insulin stimulated lipogenesis (Walton et al., 1987) whereby insulin binding of adipocytes is inhibited (Walton et al., 1986). However, in the culture of some preadipocyte cell lines, growth hormone promotes both proliferation and differentiation. For example, growth hormone enhanced proliferation and differentiation of 3T3-F442A cells (Morikawa et al., 1982;1984; Zezulak and Green, 1986) and ob 17 cells (Ailhaud et al., 1983). Zezulak and Green (1986) found that 3T3 cells synthesize IGF-1 receptors in response to growth hormone thereby increasing the sensitivity of 3T3 cells to IGF-1, a mediator of growth hormone effect. In primary culture, the effect of growth hormone is equivocal (Hausman et al., 1989). Addition of IGF-1 to rat preadipocytes primary cultures enhanced adipose conversion (Deslex et al., 1987a) but growth hormone was unable to stimulate adipose conversion (Wiederer and Loffler, 1987). Growth hormone reduced differentiation, number and size of fat cell clusters in serum free culture of porcine adipose precursor cells when compared to controls (Hausman and Martin, 1989). Recently, Kalbitz and Mueller (1990) reported that growth hormone had no effect on proliferation or differentiation of porcine preadipocytes in culture. Effect of growth hormone on development of preadipocyte cell lines are contradictory to that in primary cultures.

**Thyroid Hormone Effect on Adipocyte Development.** Triiodothyronine ($T_3$) in physiological concentrations promotes the adipose conversion of ob17 cells (Ailhaud et al., 1983; Gharbi-Chihi et al., 1984). During differentiation of ob17 cells, nuclear $T_3$ receptor number increased but without increased affinity to $T_3$ (Anselmet et al., 1984). Lipogenic enzyme activity and fatty acid synthetase mRNA were increased in ob17 cells during adipose conversion in the presence of $T_3$ addition (Gharbi-Chihi et al., 1981;1984; Vannier et al., 1985). Thyroid hormones increased fat cell number in vivo (Picon and Levacher, 1989) and stimulated proliferation and differentiation in primary culture of rat preadipocytes. $T_3$ is considered an important component in serum free medium for primary culture of adipose precursor cells of rats (Deslex et al., 1987a) and humans (Deslex et al., 1987b). However, Wiederer and Loffler (1987) found $T_3$ to be ineffective in their primary culture system of rat adipose S-V cells. The reason for this discrepancy is not known. The effect of $T_3$ in primary culture of pig S-V cells may be permissive (Hausman, 1989).

**Other Serum borne Factors.** Growth hormone has been found to be a factor in fetal calf serum (Nixon and Green, 1984) and human serum (Hauner and Loffler, 1986) promoting adipose conversion of some cell lines. There have been several reports indicating that sera contain adipogenic factors that are distinct from growth hormone. A 6-8 kDa molecule has been partially purified from fetal calf serum (Grimaldi et al., 1982). This molecule is resistant to proteolytic activity of pronase, is heat labile at low pH, and is able to induce adipose conversion of ob 17 cells (Grimaldi et al., 1982). Sera from several strains of genetically obese rodents

contain adipogenic factors with an apparent molecular weight of 4-6 kDa (Loffler et al., 1983). A similar factor has also been found to be present in human serum (Hauner and Loffler, 1986). The low molecular weight factor is heat and acid-stable (Hauner and Loffler, 1986). Feick and Loffler (1986) have extracted a 4-6 kDa molecule with adipogenic activity from rat liver. This molecule can be destroyed by pronase. Recently, a 63 kDa protein that promotes differentiation of rat preadipocytes in primary culture has been partially purified from rat serum (Li et al., 1989). The 63 kDa molecule has characteristics that are different from other growth factors in that it is unstable in acid-ethanol, unaffected by -SH reducing agents and most proteases (Li et al., 1989). The 63 kD protein may be species specific (Li et al., 1989). A 20 kD protein factor that stimulates proliferation of 3T3-L1 and OB1771 cell lines has been partially purified from rat adipose tissue and has been suggested to be different from platelet derived growth factor (PDGF) and fibroblast growth factor (FGF; Aoki et al., 1990).

In addition to adipogenic factors serum contains "anti-adipogenic" factors, some undefined (Loffler and Hauner, 1987). Transforming growth factor-ß has been shown as a serum component which inhibits the expression of differentiation enzyme in Ob1771 cells (Pradines-Figueres et al., 1990). Mitogenic factors such as PDGF and FGF inhibit differentiation of 3T3 cells (Hayashi et al., 1981; Kawada et al., 1990).

**Serum Source Effect on Adipocyte Development.** Several comparisons have been made of the ability of sera from different species and animals of various dietary

status to promote growth of preadipocytes in primary culture of adipose S-V cells. Sera from different species vary greatly in their ability to promote adipose conversion of 3T3 cell lines. Kuri-Harcuch and Green (1978) found that fetal calf serum promoted adipose conversion of 3T3-F442A cells while serum from cats did not. Serum derived from genetically obese rodents promoted substantially more adipose conversion of 3T3-L1 cells than serum from lean rodents (Loffler et al., 1983). In primary culture of rat adipocyte precursor cells all serum samples tested promoted cell proliferation, but sera from humans, calves, goats, and rats were able to increase glycerol-3-phosphate dehydrogenase activity more than did cat serum (Bjorntorp et al., 1985). Since cat can increase its body fat deposition in vivo, caution should be taken in extrapolating from in vitro studies because various growth effectors acting in opposite manner may change concurrently in vivo. The in vitro effect of cat serum may be due to differences in cells from one species to another; it is possible that cat serum contains factor(s) which inhibit adipose conversion and expression of lipogenic enzyme activity and in contrast to cat cells rat adipose precursor cells and 3T3-F442A cells possess receptors and/or promoters that make the cells more sensitive to this factors, thereby permitting interaction with the inhibiting factors. Comparisons should be made between homologous and heterologous systems since subsequent growth response in culture can be affected by source species of both cells and sera (Jewell and Hausman, 1989). From all the studies mentioned above it can be summarized that there are several factors in blood, some stimulating cell replication or differentiation, some inhibiting either or both processes, and that these factors may vary across species.

**Age Effect on Adipocyte Development.** As animals grow older several changes occur in adipose tissue mass and adipocyte function. In middle and late adulthood in human several structural changes occur. The lean body mass decreases and adipose tissue mass increases (Rudman, 1985). The body fat content of a newly born pig is less than 2% of the body weight but it increases rapidly to 30% or more of body weight at about 6 months of age (Doornenbal,1972; Shields et al., 1983). Impaired sensitivity of adipose tissue to lipogenic and lipolytic hormones such as insulin and catecholamines, and reduced glucose uptake and conversion by adipocytes have been observed in the rat as the aging process progresses (Kirkland and Dax, 1984). Bertrand et al. (1980) also observed changes in adipose tissue mass and cellularity during adult life in rats. The effect of aging on the ability of adipocyte precursor cells to replicate and differentiate in culture has been explored (Djian et al., 1983; Kirkland et al., 1990). Adipose S-V cells obtained from varying anatomical sites all showed a decline in rate of replication, and there was also a reduction in the frequency of differentiation of adipocyte precursor cells (Djian et al., 1983; Kirkland et al., 1990). This indicates that cells from younger animals replicate faster than cells from older animals and contain more clones capable of full differentiation into adipocytes. Kirkland et al. (1990) suggest that changes in gene regulation may cause the differences in both sensitivity and responsiveness of cells to hormones and paracrine factors and differences in the capacity of cells to uptake and utilize nutrients. It is not known if the age of an animal influences levels and activities of adipogenic factors present in the serum. It is therefore important to identify and characterize serum adipogenic factors so that it can be determined

whether age is a physiological factor regulating the levels and activities of serum adipogenic factors.

**Sex Effect on Adipocyte Development.** Sex of an animal influences its adiposity through endocrine profile (Seideman et al., 1982). In mammals, the intact male has less carcass fat and more muscle than the intact female, and the castrated male on the other hand has more fat and less muscle than the intact male and in some species, the female. There was increased lean tissue accretion when exogenous estrogenic compounds were administered to castrated male cattle and sheep (Schanbacher, 1984; Roche and Quirke, 1986). However, administration of exogenous estrogenic compounds showed no influence on carcass composition of pigs (De Wilde and Lauwers, 1984; Roche and Quirke, 1986). The cellular basis for some of these changes has not been examined. Examination of the growth and development of adipose tissue S-V cells culture can provide some of the cellular mechanisms involved. Also, the influence of the animal's sex on adipogenic activity of serum factors can be elucidated by using sera obtained from animals of different sex in culture of adipose S-V cells.

**Lean and Obese Pigs.** The genetically lean and obese pigs provide a model to study porcine growth, particularly deposition of carcass fat and accretion of muscle and also provide a model, other than rodent models to study the physiology of obesity. Lean and obese strains of pigs were developed by Hetzer and Harvey (1967) by selecting for thick or thin backfat depth for about eighteen generations. Obesity

can be detected in pig model as early as 100 days of gestation (McNamara and Martin, 1982; Stone et al., 1985). There is divergence in growth rate and body composition in obese and lean pigs. Obese pigs grow more slowly after weaning as compared to their lean counterparts (Mersmann et al., 1982). Fat cell number is not different in lean and obese strains of pigs (Hausman et al., 1983). Morphologically, adipocytes from obese pigs are larger than adipocytes from lean pigs (Steele et al., 1974; Scott et al., 1981; Mersmann, 1986). Larger cell size in obese pigs might contribute to their higher body lipid deposition. Adipose tissue lipogenic rates are higher in growing obese than lean pigs (Steele et al., 1974; Steele and Frobish, 1976; Scott et al., 1981).

Blood metabolites in lean and obese pigs do not differ to a great extent. Plasma glucose, triglyceride and cholesterol concentrations are similar in neonatal and postnatal obese and lean pigs (Mersmann et al., 1982). Thus, growing obese pigs are not hyperglycemic, hypertriglyceridemic or hypercholesterolemic as are other animal obesity models (Mersmann et al., 1982). Only slight differences exist in endocrine status of these pig strains. Plasma concentrations of growth hormone are lower, triiodothyronine is higher and cortisol is similar in late prenatal obese pigs as compared to lean pigs (Stone et al., 1985). Because of their unique endocrine and metabolite status the pig model of obesity is not compounded by major defects in carbohydrate and/ or lipid metabolism as are most rodent models (Mersmann et al., 1982). Thus, increase in adipose tissue mass in obese pigs might be due more to differences in the activity of the cells than to the extracellular environment. Adipocytes in lean and obese pigs are different. This belief is supported by report

of Killefer and Hu (1990) who found an adipocyte specific plasma membrane protein to be present in adipocytes of genetically lean pigs and absent in genetically obese pigs. The nature and physiological function of this protein have not been identified.

Serum from genetically obese pigs enhanced differentiation more than serum from lean pigs in primary culture of rat adipose S-V cells. It has been shown that subsequent growth response of cultured cells is influenced by species of the test S-V cells as well as the serum (Jewell and Hausman, 1989). Therefore, it is pertinent to use cells obtained from pig adipose tissue to examine the effect of lean and obese pig sera on adipose conversion in culture.

# Chapter 2

## HORMONAL REGULATION OF THE DIFFERENTIATION OF PORCINE ADIPOSE STROMAL-VASCULAR CELLS IN CULTURE

## ABSTRACT

Stromal-vascular (S-V) cells obtained by collagenase digestion of adipose tissue samples from subcutaneous neck region of 4 month old pigs (40-50 Kg) were used to evaluate the ability of S-V cells to differentiate in response to insulin, hydrocortisone (HC) and triiodothyronine ($T_3$) in culture. Cells were maintained for 12 days in test media. Insulin (1-1000 $\mu$U/ml) alone and $T_3$ (.1-100 ng/ml) did not affect sn-glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8), a marker enzyme for adipocyte differentiation. HC (500 ng/ml) alone stimulated ($P<.05$) differentiation in cultured S-V cells. Cellular protein and DNA contents of cultured cells were not affected by treatments. Cells receiving combination of HC and insulin had greater GPDH activity than cultures receiving either hormone alone. When concentrations of HC and $T_3$ were held constant in physiological ranges, increasing concentration of insulin increased ($P<.05$) both GPDH activity and DNA contents in cultures. Addition of increasing concentration of HC to cultures receiving constant levels of insulin and $T_3$ within physiological ranges had higher ($P<.05$) GPDH activity compared to the control; DNA and protein contents were not affected. Increasing concentrations of $T_3$ to cultures with constant physiological levels of insulin and HC did not change any of the parameters measured. Insulin and glucocorticoids appear essential for differentiation of porcine S-V cells in culture.

# INTRODUCTION

In vivo investigations of adipose tissue development using radiolabelled thymidine incorporation into DNA have shown that developing rat adipose tissue contains cell fractions which are able to proliferate and differentiate into mature adipocytes (Hollenberg and Vost, 1968; Greenwood and Hirsch, 1974; Gaben-Cogneville and Swierczewski, 1979). Several studies conducted with cultured stromal-vascular cells isolated from adipose tissue of various mammalian species including man provided evidence for the existence of adipose precursor cells capable of proliferating and undergoing adipose conversion (Adebonojo, 1975; Van et al., 1976; Van and Roncari, 1978; Bjorntorp et al., 1980; Ramsay et al., 1989a,b; Hauner et al., 1989). The use of several cloned cell lines as well as primary culture of adipose stromal-vascular cells in studying adipose cell growth and development has given us the knowledge and better understanding of the factors involved in the control of growth and differentiation of adipose precursor cells.

Hormones and growth factors present in the extracellular environment have profound influence on growth and development of cells. Numerous effects of hormones and growth factors on preadipocyte growth and development have been observed (Wiederer and Loffler, 1987; Roncari and Le Blanc, 1989; Hauner et al, 1989; Schmidt et al., 1990). Adipogenic activity of insulin, glucocorticoids and thyroid hormones has been clearly established (Hauner and Loffler, 1987). Insulin and glucocorticoids are essential for adipose conversion of rat adipose stromal-

vascular cells in primary culture (Gaben-Cogneville et al., 1990). As a result, a suitable system that permits detailed study of the control of adipose differentiation in rat adipose stromal-vascular cells (Deslex et al., 1987) and adipose-like cell lines (Gaillard et al., 1984; Schmidt et al., 1990; Kawada et al., 1990) maintained in a defined culture environment has been established. In contrast the hormonal control of porcine adipose precursor cell growth and development has only been partially elucidated. Hausman (1989) has demonstrated the influence of insulin, IGF-1 and triiodothyronine on the differentiation of porcine adipose-S-V cells in culture. Serum obtained from hypophysectomized pigs has lower levels of insulin-like growth factor-1 and inadequately supported growth of pig S-V cells in culture (Jewell et al., 1989). However, Ramsay et al. (1989b) and Hausman (1989) found that insulin-like growth factor-1 induced porcine preadipocyte differentiation. Ramsay et al. (1989a) have also demonstrated the requirement of glucocorticoids for the differentiation of porcine adipose S-V cells. All the pig studies mentioned either utilized cells obtained from fetal or 1-day-old pigs and some of the hormonal studies utilized hormone supplementation of serum. Therefore, in the present study we have examined the growth and development of adipose tissue stromal-vascular cells obtained from 40-50 kg pigs in the presence of insulin, triiodothyronine, and hydrocortisone and have attempted to establish a chemically defined medium to study porcine adipose stromal-vascular cell differentiation.

## MATERIALS

Dulbecco's Modified Eagle's Medium (DME, D-5523), Nutrient mixture F-12 (HAM, N-6760), dihydroxy acetone phosphate (DHAP, D-7137), reduced nicotinamide adenine dinucleotide (NADH, N-8129), gentamicin sulfate (G-1264), hydrocortisone (H-0135), insulin (I-1882), triiodothyronine (T-5516), bovine transferrin (T-8027) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA, Bovuminar Reagent CRG-7) was purchased from Armour Pharmaceutical Co. (Tarrytown, NY); collagenase (Type I) from Worthington Biochemical (Freehold, N.J.); thiamylal-sodium (Biotal) from Boehringer-Inglheim Animal Health Inc. (St. Joseph, MO); fetal calf serum (FCS) from Intergen Co. (Purchase, NY); Fungizone from Gibco BRL (Gaithersburg, MD); and Prepodyne from AMSCO, Medical Products Division (Erie, PA). All other reagents were of analytical grade.

## METHODS

### Animal and biopsy procedure

Crossbred barrows (*Sus scrofa*) weighing 40-50 kg were used in this study. Before biopsy pigs were scrubbed with Prepodyne and subcutaneous adipose tissue samples were obtained as described by Akanbi et al. (1990).

Stromal-vascular cell isolation

Tissue samples were transported to the laboratory in Krebs-Ringer bicarbonate buffer (KRB, 37 °C, pH 7.4) containing 118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.3 mM Mg $SO_4$, 10 mM $NaHCO_3$, 10 mM HEPES, 5 mM glucose and 40 mg/L gentamicin sulfate and equilibrated with 95% $O_2$:5% $CO_2$. Adipose tissue samples were digested at 37 °C in a gyratory water bath with 2 mg/ml collagenase in KRB buffer containing 3% BSA for 1 hr (1 g tissue per 3 ml media). Digested tissue was filtered through a sterile single layer of polyester chiffon and cell suspension was centrifuged at 800 X g for 10 min. Stromal-vascular (S-V) cell pellets were washed three times in DME/HAM medium (1:1; v/v) containing 15 mM $NaHCO_3$, 15 mM HEPES buffer (pH 7.4), 40 mg/L gentamicin sulfate and 2 mg/L Fungizone (basal medium).

Cell Culture

Aliquots of the S-V cells were removed, stained with Rappaport's stain and counted on a hemocytometer. S-V cells were seeded in basal medium containing 10% FCS on Corning 6 well (35mm) tissue culture plates at a density of $3 \times 10^4$ cells/$cm^2$. Cells were cultured at 37 °C under a humidified atmosphere of 95% air:5% $CO_2$; 24 hours later cells were washed 2 x 5 minutes and 1 x 1 hour with basal medium. Cells were subsequently cultured in basal medium containing 10% FCS until they became confluent. After confluence, cells were washed as described above and cultured in basal medium supplemented with different hormones as indicated in figures and legends. Medium was changed every 3 days until day 12

(post-confluence) when protein, DNA and sn-glycerol-3-phosphate dehydrogenase (GPDH) activity were determined. GPDH activity has been reported to rise after lag phase and maximum activity is reached about 20 days (Schmidt et al., 1990).

Enzyme Analysis

Sn-glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) was measured by a spectrophotometric method for determination of disappearance of NADH during GPDH-catalyzed reduction of DHAP under zero order conditions (Kozak and Jensen, 1974) as modified by Wise and Green (1979).

DNA and Protein Content

DNA was assayed as described by LaBarca and Paigen (1980) using salmon testes DNA as a standard. Protein content was determined by bicinchoninic acid (BCA) method using bovine serum albumin as a standard (Pierce Chemical Co., Rockford, IL).

Histochemistry

Representative wells from each treatment were fixed in 10% formalin, stained with oil red O to show lipid droplets and counterstained with Harris hematoxylin (Boon and Drijver, 1986) after 12 d of exposure to test media.

Statistical Analysis

The data obtained were subjected to one-way analysis of variance procedure of NCSS (1984). Differences between means were determined by Fisher's LSD.

# RESULTS

Stromal-vascular cells obtained from adipose tissue from subcutaneous neck region of pigs weighing 40-50 kg inoculated at a density of $3 \times 10^4$ cells/cm$^2$ and cultured in DME/HAM's medium supplemented with 10% FCS, cells grown in this medium reached confluence after 6 days. In the first series of experiments the effect of increasing concentrations of insulin, hydrocortisone and triiodothyronine ($T_3$) on differentiation of confluent cells were tested. Figure 2.1 shows the effect of increasing concentrations of insulin on GPDH activity, protein and DNA contents of cultured porcine adipose S-V cells. Cellular protein content or DNA content of cultures was not altered ($P > .05$) by increasing concentrations of insulin. The data indicate that insulin did not affect ($P > .05$) GPDH activity either expressed on per well basis or per unit protein basis.

Figure 2.2 shows the effect of increasing concentrations of hydrocortisone on GPDH activity, cellular protein and DNA contents of cultured porcine S-V cells. Protein and DNA contents of cultures treated with different concentrations of hydrocortisone were not significantly altered ($P > .05$). Hydrocortisone (500 ng/ml) stimulated GPDH activity significantly ($P < .05$). Stimulation of GPDH activity by 500 ng/ml hydrocortisone accounted for over a 100% increase when compared to cultures without added hormones. Hydrocortisone at concentrations lower than 500 ng/ml had no effect on GPDH activity ($P > .05$).

Effects of increasing concentrations of $T_3$ on GPDH activity, cellular protein and DNA contents are shown in Figure 2.3. Addition of increasing concentrations

of $T_3$ did not change protein or DNA content of cultured cells or GPDH specific activity ($P > .05$).

Insulin did not increase GPDH activity at a nonsignificant rate (Fig. 2.1), and very little or no cytoplasmic lipid accumulation was observed (Fig. 2.4b). Morphological changes were observed in cultures treated with hydrocortisone. After the addition of insulin to confluent cells, the cells assume a rounded shape with the cytoplasm protruding in different directions instead of the long spindle-like shape they have prior to the addition of hydrocortisone (Fig. 2.4c). Little cytoplasmic lipid was observed in cultures treated with 500 ng/ml hydrocortisone (Fig. 2.4c). The shape of the cells in culture treated with $T_3$ remained spindle-like like the control and there was no visible cytoplasmic lipid (Fig. 2.4).

Using insulin (100 $\mu$U/ml), hydrocortisone (500 ng/ml) and $T_3$ (1 ng/ml), possible interactions among the hormones to promote S-V cell differentiation were examined. During a culture period of 12 days after confluence in insulin and hydrocortisone containing media, no significant increase in cellular protein level occurred and DNA level did not change (Table 2.1). Insulin and hydrocortisone combined accelerated both lipid accumulation (Fig. 2.5) and GPDH activity (Table 2.1) in cultured S-V cells. Insulin in combination with hydrocortisone increased GPDH activity by over 100% as compared to insulin or hydrocortisone alone (Table 2.1; $P < .05$). DNA content of cultured cells was not affected by combination of $T_3$ and insulin (Table 2.1). However, combination of $T_3$ and insulin increased GPDH activity over 100% as compared to insulin or $T_3$ alone (Table 2.1).

Although the data indicate that GPDH activity in cultures treated with the

combination of $T_3$ and hydrocortisone doubled GPDH activity, this was not statistically significant (Table 2.1). This might be as a result of varied plating efficiency and cell detachment from culture to culture. In the presence of insulin, cultures treated with $T_3$ showed very little cytoplasmic lipid (Fig. 2.5b). $T_3$ and hydrocortisone did not interact to stimulate lipid accumulation in cultured cells (Fig. 2.5c).

In the next series of experiments levels of two hormones were held constant at physiological concentrations and variable concentrations of the third hormone were added to confluent porcine adipose S-V cells. Table 2.2 shows the effect of increasing concentration of insulin on GPDH activity, protein and DNA contents in cultured cells when $T_3$ and hydrocortisone concentrations were held constant at physiological levels. Only cultures with 100 and 1000 $\mu$U/ml insulin had significant increases in cellular protein levels as compared to $T_3$ and hydrocortisone alone ($P<.05$). A low level of insulin (1 $\mu$U/ml) did not significantly affect DNA content of cultured cells ($P>.05$). Ten $\mu$U/ml of insulin significantly increased DNA level ($P<.05$) as compared to cultures without insulin (with $T_3$ and hydrocortisone). There was also significant increase in DNA in cultures supplemented with 100 and 1000 $\mu$U/ml insulin ($P<.01$). When $T_3$ and hydrocortisone at physiological concentrations were used there was no effect on lipid accumulation of confluent cells. With increasing concentrations of insulin, a modest increase in lipid accumulation was observed. Insulin at concentrations of 1 and 10 $\mu$U/ml significantly increased GPDH activity in cultured cells ($P<.01$; Table 2.2). At insulin concentrations of 100 and 1000 $\mu$U/ml there was a slight drop in GPDH activity.

However, addition of 100 $\mu$U/ml of insulin still produced a significantly higher GPDH activity than $T_3$ and hydrocortisone alone ($P<.05$).

When the concentrations of insulin and hydrocortisone were held constant at physiological levels and variable concentrations of $T_3$ were added (Table 2.3), neither protein nor DNA levels was significantly affected and there was no significant change in GPDH activity of cultured cells ($P>.05$). When the concentrations of insulin and $T_3$ were held constant at physiological levels addition of variable concentrations of hydrocortisone did not alter cellular protein and DNA levels in cultured cells (Table 2.4). Holding the concentrations of insulin and $T_3$ constant at physiological ranges and adding variable concentrations of hydrocortisone did alter GPDH activity in cultured cells (Table 2.4). Five ng/ml of hydrocortisone did not significantly change GPDH activity ($P>.05$); however, at higher concentrations (50 and 500 ng/ml) hydrocortisone significantly increased GPDH activity in cultured porcine S-V cells ($P<.05$).

## DISCUSSION

The hormonal requirements for differentiation of S-V cells isolated from adipose tissue (rodents and human) have received considerable attention (Hauner and Loffler, 1987; Loffler and Hauner, 1987; Wiederer and Loffler, 1987). In contrast the hormonal requirements for adipose conversion of porcine S-V cells has been only partially elucidated. In the present study we have attempted to examine the adipogenic and biochemical differentiating effects of insulin, hydrocortisone and $T_3$ alone or in combinations. Primary cultures from S-V cells derived from collagenase digestion of adipose tissue have been used to study adipocyte development from precursor cells (Gaben-Cogneville et al, 1983; 1984; Deslex et al., 1987; Hausman, 1989; Hauner et al., 1989). These present studies used S-V cells obtained from 3-4 month old pigs (40-50 kg). Cells were cultured in basal medium plus 10% FCS for about 6 days until confluence, after which hormonal treatments were applied for another 12 days. GPDH, a marker enzyme for adipose differentiation, (Hauner et al., 1989; Wiederer and Loffler, 1987; Gaben-Cogneville et al. 1990) was used as an indicator of the differentiation activity. Increase in GPDH activity during differentiation is dependent on seeding density and starts only when cells become confluent (Petterson et al., 1985). Significant differentiation of rat adipose S-V cells was observed when cells became confluent (Petterson et al., 1985). Adding insulin alone to confluent cells did not significantly affect differentiation activity in porcine S-V cells. This result is in agreement with the

work of Hentges and Hausman (1989) who reported that insulin did not influence GPDH activity in cultures of S-V cells obtained from 1 day old pigs. The differentiation of adipose S-V cells obtained from various species has been found to be under the control of several hormonal factors. The influence of insulin on the differentiation of S-V cells obtained from rats (Gaben-Cogneville et al., 1983; 1984; Wiederer and Loffler, 1987) and 1 day old pigs (Hausman, 1989) has been demonstrated. Gaben-Cogneville et al. (1983; 1984) found that insulin (at physiological ranges) alone can stimulate complete differentiation of newborn rat adipocyte precursor cells and Hausman (1989) who found that insulin stimulated formation of fat clusters from S-V cells of 1 day old pigs. In contrast, in the current study insulin alone had no effect on lipid accumulation (Fig. 2.4b) or GPDH activity (Table 2.1). Hormonal treatments were imposed after confluency in our studies and the study of Hausman (1989) but S-V cells were obtained from pigs of differing ages (1 day *vs* 4 months). It is not known whether the culture conditions used are the bases for the conflicts in results between the study by Hausman (1989) and Hentges and Hausman (1989). Others (Gaben-Cogneville et al., 1983; 1984; Wiederer and Loffler, 1987) used newborn or young rats and started their treatments before S-V cells reached confluence. It is likely that signals required to trigger the differentiation processes in S-V cells from older animals might have been arrested prior to the addition of hormones. Li et al. (1989) did not observe insulin effect on the differentiation of S-V cells from older rats and attributed the differences between their observations and the insulin-promoted differentiation of S-V cells from adipose tissue of newborn and young rats (Gaben-Cogneville et

al.,1984; Wiedere and Loffler, 1987) in parts to age differences. The variations in the results of the present study might be due to age differences and/or culture condition imposed. Age-dependency of S-V cell differentiation has been observed (Bjorntorp et al., 1980; Djian et al., 1985; Deslex et al., 1987; Hauner et al., 1989). Since insulin alone did not change the cellular protein and DNA levels in cultured cells , these observations imply that insulin alone does not promote changes in cell number or size. Regulation of GPDH by insulin is at the transcriptional level (Gaben-Cogneville et al., 1990); the modest but not statistically significant increase in GPDH activity by insulin might be due to low level of GPDH mRNA as a result of decreased capacity of cells obtained from older animal to respond to extracellular stimuli (Hauner et al., 1989). Hydrocortisone alone at concentration of 500 ng/ml (Fig. 2.2) stimulated high GPDH activity but did not accelerate accumulation of cytoplasmic lipid droplets in cultured cells (Fig. 2.4). Hydrocortisone did not promote cellular growth as indicated by protein and DNA levels in the cultures (Fig. 2.2). Hydrocortisone in combination with insulin, however, accelerated both lipid accumulation (Fig. 2.5) and GPDH activity (Table 2.1). Rapid increases in GPDH gene expression and GPDH activity have been observed in rat primary cultures treated with dexamethasone, a synthetic glucocorticoid (Gaben-Cogneville et al., 1990). It has also been suggested that glucocorticoids have an effect similar to insulin on the expression of lipogenic enzymes and on increasing insulin binding of cultured cells (Gaben-Cogneville et al., 1990). As a result, the effect of interaction of insulin and hydrocortisone on the marker enzyme in the present studies appears to be synergistic. The present observation on the effect of combination of insulin

and hydrocortisone is not limited to primary cultures, this combination of hormones has been shown to promote adipogenic conversion of clonal cell line, TA1 (Chapman et al., 1984). The combination of these two hormones trigger developmental programs that allow the cells to convert from preadipocytes to mature adipocytes (Chapman et al., 1984) Adipose conversion of TA1 cells by insulin depends on the presence of glucocorticoid (Chapman et al., 1985). One of the major adipogenic factors in serum has been identified as glucocorticoids (Schiwek and Loffler, 1987). The present results on insulin and hydrocortisone are in agreement with reports by Wiederer and Loffler (1987) and Hauner et al.(1987) that insulin plus corticosterone stimulate adipose conversion and differentiation of S-V cells from rat and human. Quantitative response of GPDH activity in S-V cells to treatments (Table 2.1) is, however, higher than response in tables 2.2, 2.3 and 2.4. The pigs that served as cell donors for the experiment in table 2.1 were 3 weeks younger than those used for the experiments in the other studies. The source of cells might contribute to the quantitatively higher GPDH activity observed for this particular experiment (Table 2.1). Animal age influences the subsequent growth responses of S-V cells in culture (Bjorntorp et al., 1980; Djian et al., 1985; Deslex et al., 1987; Hauner et al., 1989; present study). Insulin and glucocorticoids act as adipogenic factors in S-V cells regardless of the animal species the cells are obtained from. None of the concentrations of $T_3$ used in the present studies had any effect on GPDH activity of cultured porcine S-V cells. $T_3$ has been found to be ineffective in serum free cultures (Deslex et al., 1987) and its effect on adipose conversion may be permissive and requires the presence of other hormones

(Hausman, 1989). Only in the presence of insulin did cultures treated with $T_3$ show lipid accumulation (Fig. 2.5). Combination of $T_3$ and hydrocortisone did not significantly change the activity of the enzyme marker, GPDH in cultured S-V cells (Table 2.1). The lack of statistical significance in the increased GPDH activity of cultures treated with $T_3$ plus hydrocortisone as compared to hydrocortisone alone (Table 2.1) might be in part due to varied plating efficiency and cell detachment from experiment to experiment.

By holding concentrations of $T_3$ and hydrocortisone constant at physiological range, addition of increasing concentrations of insulin showed mitogenic and adipogenic effect as indicated by increase in GPDH activity and DNA contents in cultured cells (Table 2.2). Increase in DNA might be a reflection of increased cell number. Duliere et al., (1991) reported that addition of various concentrations of insulin to porcine S-V cell culture promoted cell proliferation. Several possibilities could explain the stimulation of cell proliferation by insulin. One possibility is that insulin at pharmacological level could bind to the high-affinity receptor of IGF-1 (Hausman et. al., 1989). Clemmons (1985) found that insulin may stimulate the secretion of somatomedin-like peptide in cultured porcine smooth muscle cells. Somatomedin-like peptide can stimulate DNA synthesis in cultured human fibroblast and porcine smooth muscle cells (Clemmons and Van Wyk, 1985). The increase in DNA content of cultured porcine S-V cells by insulin might be due to either of the possibilities or both. Higher concentrations of insulin also promoted cellular growth as indicated by the significant increase in cellular protein level. In the presence of physiologic concentration of insulin and hydrocortisone (Table 2.3), addition of

various concentrations of $T_3$ did not result in significant increase in differentiation activity in S-V cells. DNA and protein levels in cultured cells were not affected by increasing concentrations of $T_3$ indicating that cellular growth was not promoted. Results in Table 2.4, however, indicate that glucocorticoids are indeed of great importance in the differentiation of porcine S-V cells. Glucocorticoids have been previously found to be a major component of adipogenic factors in serum (Schiwek and Loffler, 1987). Results in Table 2.2 shows that insulin can increase DNA content in cultured cells, however, in the experiments where insulin concentration was held constant at 20 $\mu$U/ml (Tables 2.3 and 2.4), cellular DNA content did not increase, indicating that insulin may have an optimum concentration above which it can stimulate in DNA synthesis.

The present studies demonstrate that insulin and glucocorticoids are essential for the differentiation of porcine adipose S-V cells in culture and that adipose tissue of older animals contains cells that are able to proliferate and to develop the adipocyte phenotype when cultured under the appropriate conditions. These cultured cells can be a tool to study adipocyte development in adult life.

Table 2.1. HORMONAL CONTROL OF GPDH ACTIVITY, PROTEIN AND DNA IN CULTURED PORCINE ADIPOSE STROMAL-VASCULAR CELLS[1]

| Treatment | n | GPDH[2] | Protein (μg/well) | DNA (μg/well) |
|---|---|---|---|---|
| Insulin (Ins) | 3 | 12.47 ± 1.87[b] | 318.63 ± 5.39[a] | .90 ± .09[ab] |
| Hydrocortisone (HC) | 3 | 9.93 ± 3.9[b] | 340.10 ± 22.43[a] | .51 ± .08[a] |
| $T_3$ | 3 | 14.83 ± 1.49[b] | 351.37 ± 10.94[a] | .69 ± .08[ab] |
| Ins X HC | 5 | 28.67 ± 6.18[a] | 359.91 ± 32.64[a] | 1.4 ± .18[b] |
| Ins X $T_3$ | 5 | 28.36 ± 1.22[a] | 310.07 ± 19.39[a] | 1.06 ± .18[b] |
| $T_3$ X HC | 5 | 18.93 ± 3.79[ab] | 328.61 ± 15.54[a] | .82 ± .19[ab] |

Table 2.1 Continued.

Cells were seeded at a density of $3x10^4$ cells/$cm^2$ in DME/HAM medium supplemented with 10% FCS until they were confluent. Cells were extensively washed in DME/HAM medium without FCS and subsequently maintained in DME/HAM containing 10 $\mu$g/ml transferrin plus maximally effective concentration of insulin (100 $\mu$U/ml), HC (25 ng/ml) or $T_3$ (1 ng/ml) alone or in combinations. GPDH activity, protein and DNA contents were determined at day 12 post-confluence and used as indicators of cell differentiation and growth.

[1]Data are means ± SE of n different cultures from triplicate wells.

[2]Nanomoles/minute/milligram protein.

[abcd]Means within a column that do not have a common superscript differ ($P < .05$).

Table 2.2. INSULIN EFFECT ON GPDH ACTIVITY, PROTEIN AND DNA IN CULTURED PORCINE ADIPOSE STROMAL-VASCULAR CELLS[1]

| Treatment | n | GPDH[2] | Protein (μg/well) | DNA (μg/well) |
|---|---|---|---|---|
| TTC[3] Alone | 3 | 1.94 ± .44[a] | 343.23 ± 9.85[a] | .77 ± .03[a] |
| + 1 μU/ml Insulin | 3 | 6.52 ± 1.08[bc] | 393.23 ± 23.02[ac] | .96 ± .08[ac] |
| + 10 μU/ml Insulin | 3 | 6.52 ± 1.31[bc] | 400.53 ± 5.42[ac] | 1.21 ± .15[bc] |
| + 100 μU/ml Insulin | 3 | 5.2 ± .49[bc] | 443.43 ± 17.00[bc] | 1.36 ± .05[b] |
| + 1000 μU/ml Insulin | 3 | 4.58 ± .51[ac] | 451.53 ± 43.88[bc] | 1.53 ± .17[b] |

Table 2.2 Continued.

Cells were seeded at a density of $3x10^4$ cells/$cm^2$ in DME/HAM medium supplemented with 10% FCS until they were confluent. Cells were extensively washed in DME/HAM medium without FCS and subsequently maintained in DME/HAM containing constant concentrations of three factors (concentration within the physiological range) and variable concentrations of the fourth (concentrations ranging from physiological to pharmacological levels) as indicated. GPDH activity, protein and DNA contents in cultured cells on day 12 after changing to experimental media were used as indicators of cell differentiation and growth.

[1]Data are means ± SE of three different cultures from triplicate wells.

[2]Nanomoles/minute/milligram protein.

[3]T = 10$\mu$g/ml transferrin; T = 1ng/ml $T_3$; C = 25 ng/ml hydrocortisone.

[abc]Means within a column that do not have a common superscript differ ($P<.05$).

Table 2.3. TRIIODOTHYRONINE EFFECT ON GPDH ACTIVITY, PROTEIN AND DNA IN CULTURED PORCINE ADIPOSE STROMAL-VASCULAR CELLS[1]

| Treatment | n | GPDH[2,3] | Protein[3] (μg/well) | DNA[3] (μg/well) |
|---|---|---|---|---|
| ITC[4] Alone | 3 | 7.12 ± 2.38 | 392.19 ± 48.76 | 1.20 ± .12 |
| + .1 ng/ml $T_3$ | 3 | 5.65 ± 1.10 | 439.20 ± 10.72 | 1.18 ± .03 |
| + 1 ng/ml $T_3$ | 3 | 5.63 ± 1.60 | 436.45 ± 11.36 | 1.23 ± .04 |
| + 10 ng/ml $T_3$ | 3 | 5.54 ± 1.15 | 437.10 ± 11.41 | 1.31 ± .03 |
| + 100 ng/ml $T_3$ | 3 | 4.81 ± 1.33 | 456.48 ± 33.33 | 1.25 ± .12 |

Table 2.3 Continued

Cells were seeded at a density of $3x10^4$ cells/$cm^2$ in DME/HAM medium supplemented with 10% FCS until they were confluent. Cells were extensively washed in DME/HAM medium without FCS and subsequently maintained in DME/HAM containing constant concentrations of three factors (concentration within the physiological range) and variable concentrations of the fourth (concentrations ranging from physiological to pharmacological levels) as indicated. GPDH activity, protein and DNA contents in cultured cells on day 12 after changing to experimental media were used as indicators of cell differentiation and growth.

[1]Data are means ± SE of three different cultures from triplicate wells.

[2]Nanomoles/minute/milligram protein.

[3]No significant difference between treatments at $P<.05$.

[4]I = 20 $\mu$U/ml insulin; T = 10 $\mu$g/ml transferrin; C = 25 ng/ml hydrocortisone.

Table 2.4. HYDROCORTISONE EFFECT ON GPDH ACTIVITY, PROTEIN AND DNA IN CULTURED PORCINE ADIPOSE STROMAL-VASCULAR CELLS[1]

| Treatment | n | GPDH[2] | Protein (μg/well) | DNA (μg/well) |
|---|---|---|---|---|
| ITT[3] Alone | 3 | 2.21 ± .42[a] | 384.85 ± 28.49[a] | 1.26 ± .14[a] |
| + 5 ng/ml HC[4] | 3 | 2.63 ± .45[a] | 409.32 ± 26.25[a] | 1.35 ± .11[a] |
| + 50 ng/ml HC | 3 | 8.16 ± 2.32[b] | 432.97 ± 22.55[a] | 1.21 ± .05[a] |
| + 500 ng/ml HC | 3 | 9.56 ± 2.23[b] | 435.13 ± 14.48[a] | 1.18 ± .05[a] |

Table 2.4 Continued.

Cells were seeded at a density of $3x10^4$ cells/$cm^2$ in DME/HAM medium supplemented with 10% FCS until they were confluent. Cells were extensively washed in DME/HAM medium without FCS and subsequently maintained in DME/HAM containing constant concentrations of three factors (concentration within the physiological range) and variable concentrations of the fourth (concentrations ranging from physiological to pharmacological levels) as indicated. GPDH activity, protein and DNA contents in cultured cells on day 12 after changing to experimental media were used as indicators of cell differentiation and growth.

[1]Data are means ± SE of three different cultures from triplicate wells.

[2]Nanomoles/minute/milligram protein.

[3]I = 20 $\mu$U/ml insulin; T = 10 $\mu$g/ml transferrin; T = 1 ng/ml $T_3$

[4]HC = Hydrocortisone

[abc]Means within a column that do not have a common superscript differ ($P<.05$).

FIGURE 2.1. *(A) Dose-response relationship of insulin to the activity of enzyme marker of adipocyte differentiation. (B) Dose-response effect of insulin on protein and (C) on DNA contents of cultured porcine adipose stromal-vascular cells.* Cells were inoculated on 6 well plate at a density of $3 \times 10^4$ cells/cm$^2$ in DME/HAM medium supplemented with 10% FCS until they were confluent. Cells were extensively washed in DME/HAM medium without FCS and subsequently maintained in DME/HAM containing 10 $\mu$g/ml transferrin (control) or variable concentrations of insulin (1-1000 $\mu$U/ml). GPDH activity, cellular protein and DNA contents in cultured cells was determined at d 12 post-confluence. GPDH activity was used as indicator of cell differentiation. Cellular protein and DNA were used as indicators of cell number and cellular growth. There was no significant treatment effects on GPDH activity, cellular protein and DNA contents of cultured cells ($P > .05$). Values are means ± SE of three independent experiments performed on triplicate wells. Means with the same letter are not significantly different at ($P < .05$).

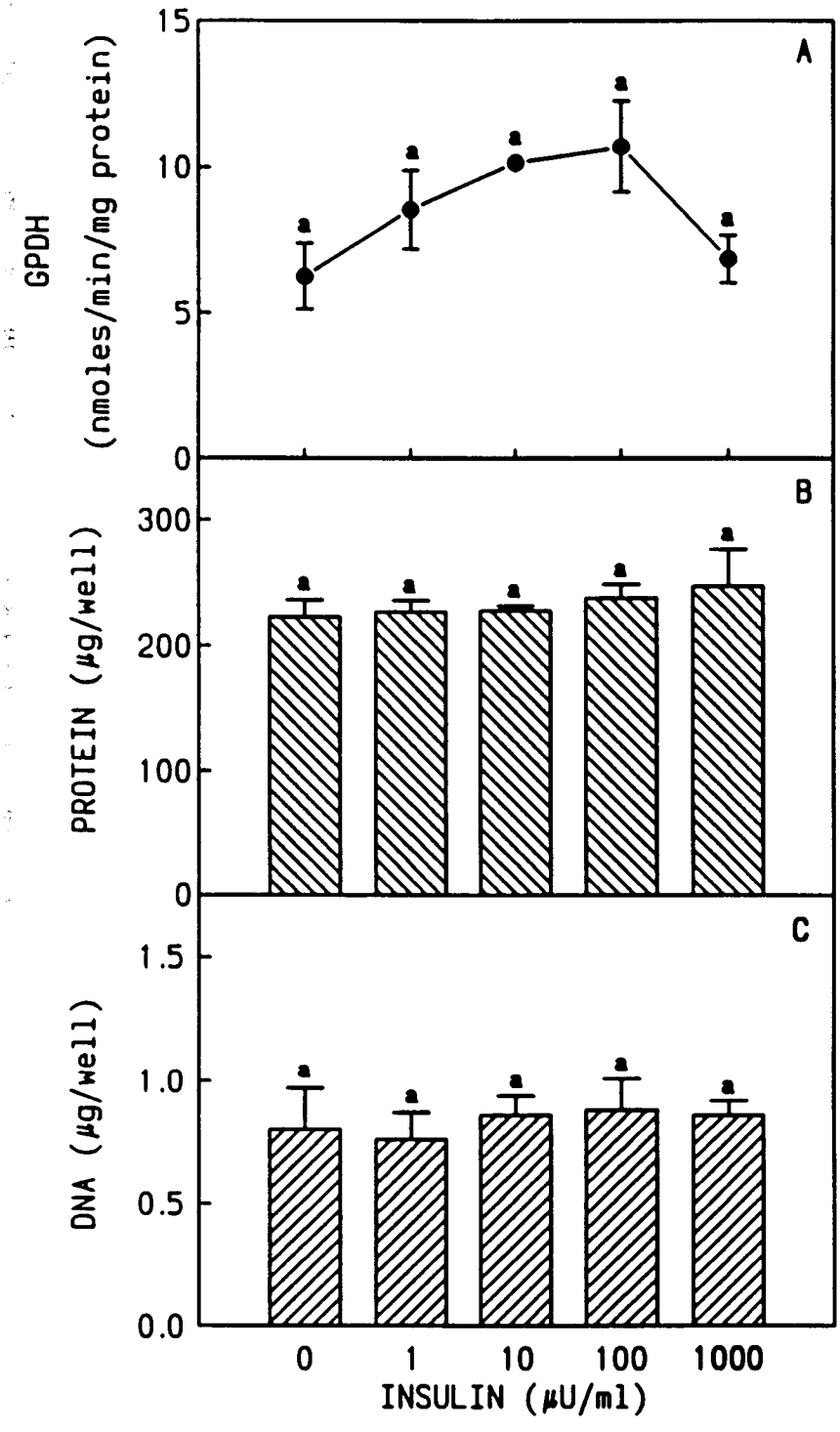
GPDH
(nmoles/min/mg protein)
15
10
5
0
A
PROTEIN (μg/well)
300
200
100
0
B
DNA (μg/well)
1.5
1.0
0.5
0.0
C
0
1
10
100
1000
INSULIN (μU/ml)

Figure 2.1

FIGURE 2.2. *(A) Dose-response relationship of hydrocortisone to the activity of enzyme marker of adipocyte differentiation. (B) Dose-response effect of hydrocortisone on protein and (C) on DNA contents of cultured porcine adipose stromal-vascular cells.* Cells were inoculated on 6 well plate at a density of $3 \times 10^4$ cells/$cm^2$ in DME/HAM medium supplemented with 10% FCS until they were confluent. Cells were extensively washed in DME/HAM medium without FCS and subsequently maintained in DME/HAM containing 10 $\mu$g/ml transferrin (control) or variable concentrations of hydrocortisone (5-500 ng/ml). GPDH activity, cellular protein and DNA contents in cultured cells were determined at d 12 post-confluence. GPDH activity was used as indicator of cell differentiation. Cellular protein and DNA were used as indicators of cell number and cellular growth. 500 ng/ml hydrocortisone significantly increased GPDH activity ($P < .05$). There was no significant treatment effects on cellular protein and DNA contents of cultured cells ($P > .05$). Values are means ± SE of three independent experiments performed on triplicate wells. Means with the same letter are not significantly different at ($P < .05$).

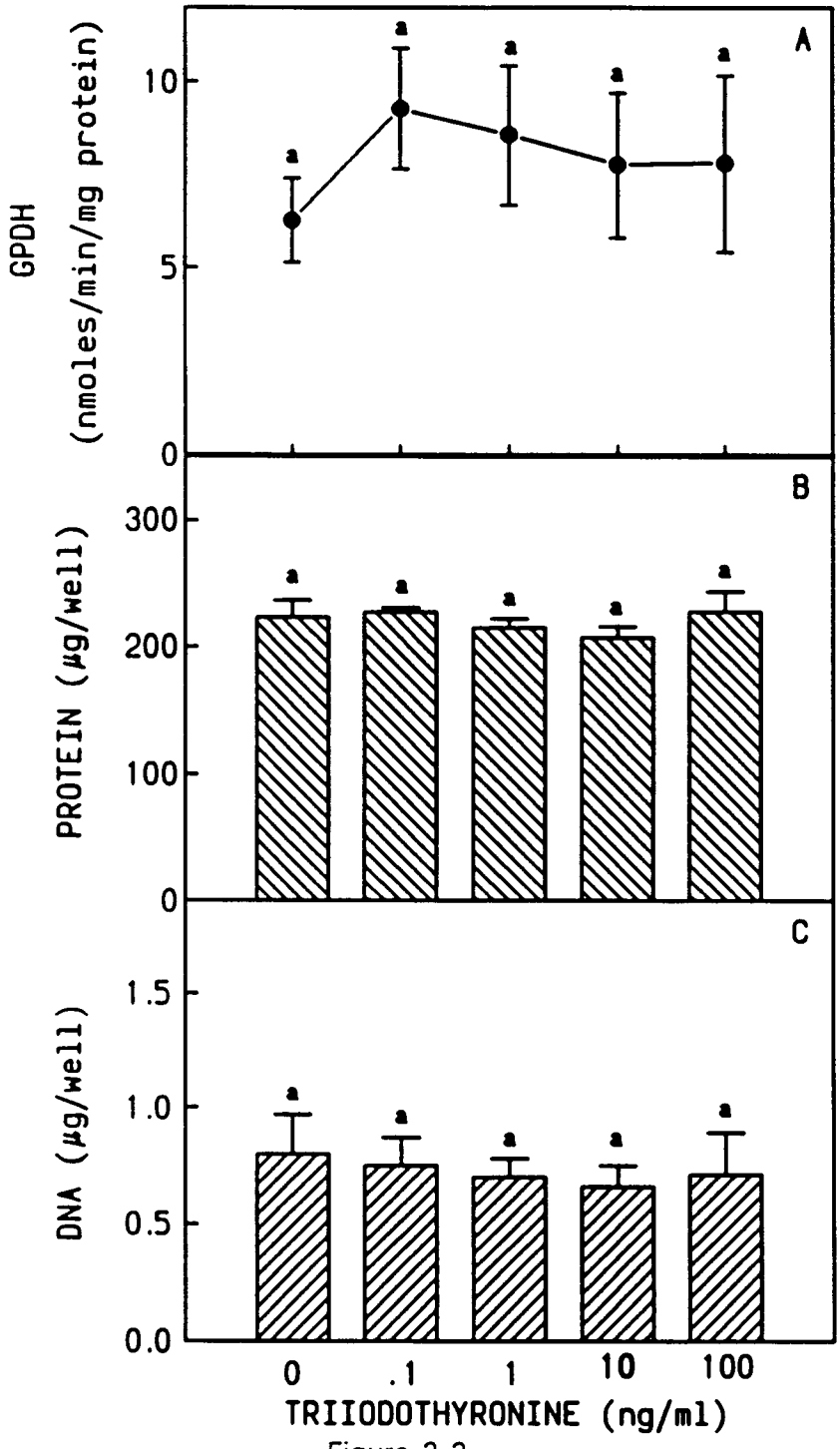
A
B
C
GPDH
(nmoles/min/mg protein)
PROTEIN (µg/well)
DNA (µg/well)
10
5
0
300
200
100
0
1.5
1.0
0.5
0.0
a
0
.1
1
10
100
TRIIODOTHYRONINE (ng/ml)

Figure 2.2

FIGURE 2.3. *(A) Dose-response relationship of triiodothyronine to the activity of enzyme marker of adipocyte differentiation. (B) Dose-response effect of triiodothyronine on protein and (C) on DNA contents of cultured porcine adipose stromal-vascular cells.* Cells were inoculated on 6 well plate at a density of $3 \times 10^4$ cells/$cm^2$ in DME/HAM medium supplemented with 10% FCS until they were confluent. Cells were extensively washed in DME/HAM medium without FCS and subsequently maintained in DME/HAM containing 10 $\mu$g/ml transferrin (control) or variable concentrations of triiodothyronine (.1-100 ng/ml). GPDH activity, cellular protein and DNA contents in cultured cells was determined at d 12 post-confluence. GPDH activity was used as indicator of cell differentiation. Cellular protein and DNA were used as indicators of cell number and cellular growth. There was no significant treatment effects on GPDH activity, cellular protein and DNA contents of cultured cells ($P > .05$). Values are means ± SE of three independent experiments performed on triplicate wells. Means with the same letter are not significantly different at ($P < .05$).

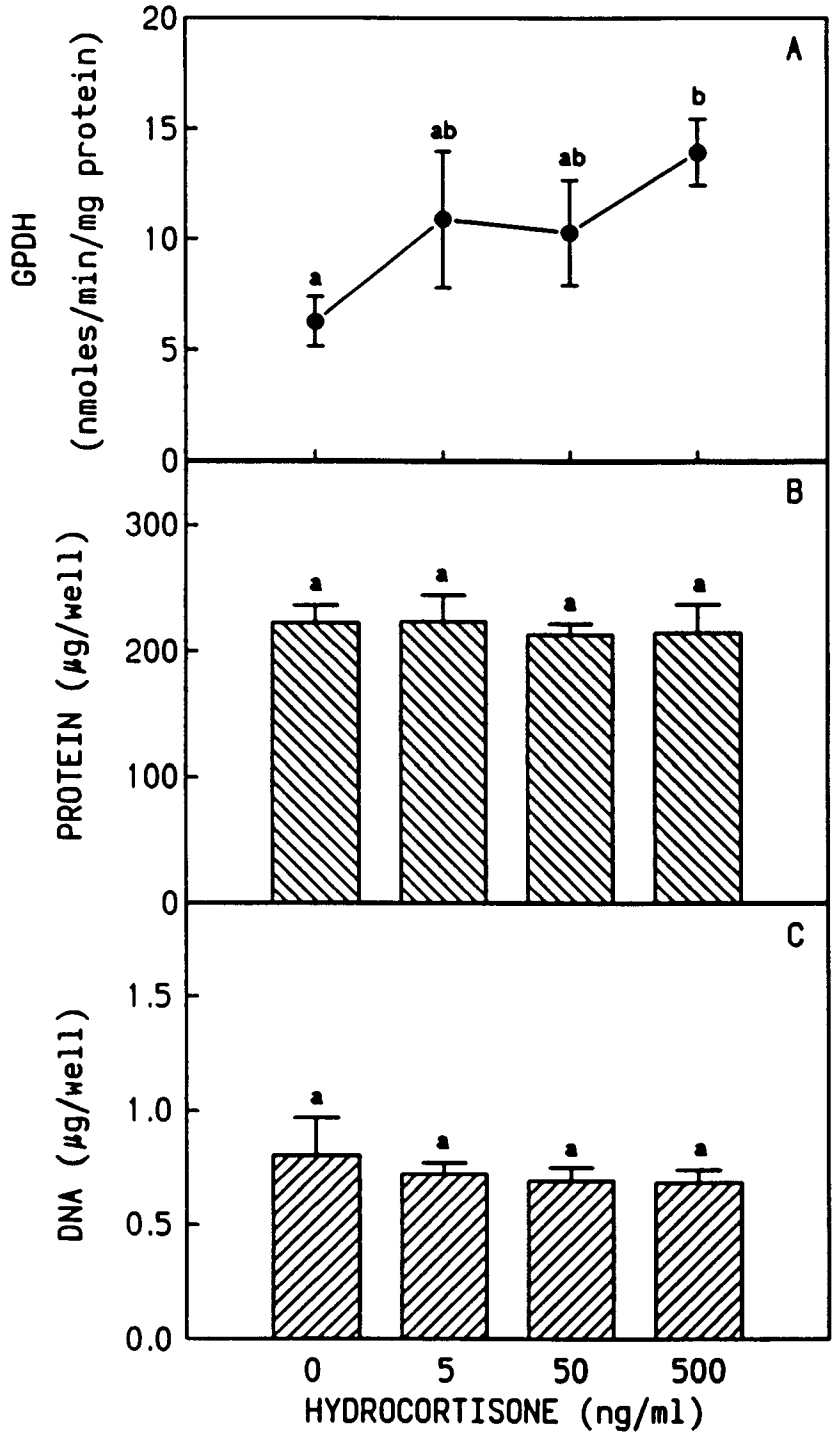
GPDH
(nmoles/min/mg protein)
A
20
15
10
5
0
a
ab
ab
b
PROTEIN (µg/well)
B
300
200
100
0
a
a
a
a
DNA (µg/well)
C
1.5
1.0
0.5
0.0
a
a
a
a
0
5
50
500
HYDROCORTISONE (ng/ml)

Figure 2.3

FIGURE 2.4. *Photomicrographs of confluent porcine adipose stromal-vascular cells stained with oil red O.* Post-confluent cells were cultured for 12 d in (A) DME/HAM medium containing 10 $\mu$g/ml transferrin without hormone supplementation (a) or with 100 $\mu$U/ml insulin (b), 500 ng/ml hydrocortisone (c), 1 ng/ml triiodothyronine (d). Cells were washed in phosphate buffered saline, fixed in 10% formalin, stained with oil red O and counterstained with Harris hematoxylin. Cells stained with oil red indicate lipid deposition. Nuclei were stained blue. None of the treatments promoted lipid deposition. Magnification = 100x. Bar = 200 $\mu$.

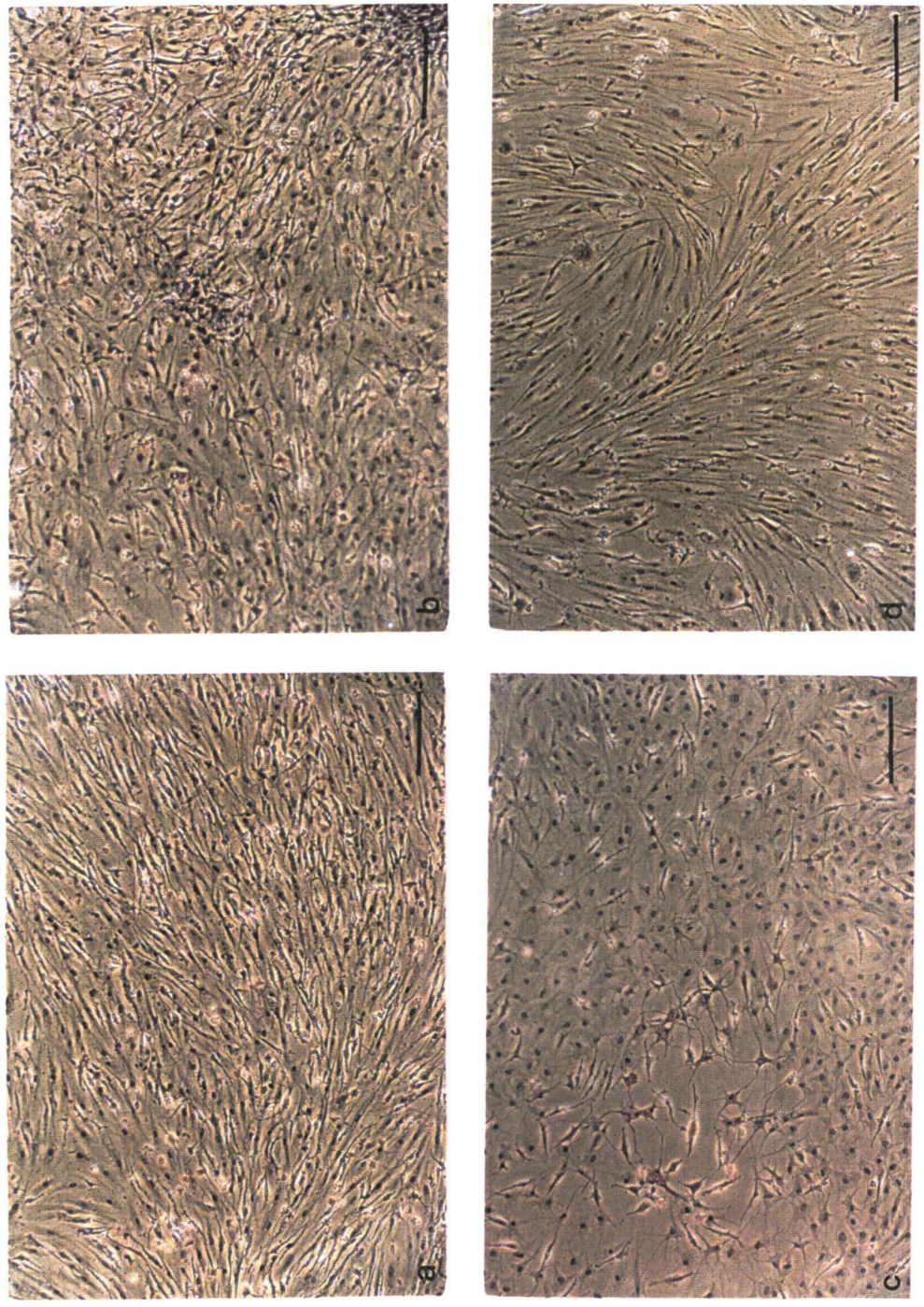


Figure 2.4

FIGURE 2.5. *Photomicrographs of confluent porcine adipose stromal-vascular cells treated with combinations of different hormones.* Post-confluent cells were cultured for 12 d in DME/HAM medium containing 10 $\mu$g/ml transferrin; supplemented with (A) 100 $\mu$U/ml insulin and 500 ng/ml hydrocortisone, (B) 1 ng/ml triiodothyronine and 100 $\mu$U/ml insulin and (C) 500 ng/ml hydrocortisone and 1 ng/ml triiodothyronine. Cells were washed in phosphate buffered saline, fixed in 10% formalin, stained with oil red O and counterstained with Harris hematoxylin. Cytoplasmic lipid droplets were stained red with oil red O and indicated lipid deposition. Cell nuclei were stained blue with hematoxylin.

Combination of insulin and hydrocortisone stimulated lipid deposition. Lipid deposition was seen in cultures treated with triiodothyronine only when insulin was present. Magnification = 100x. Bar = 200 $\mu$.

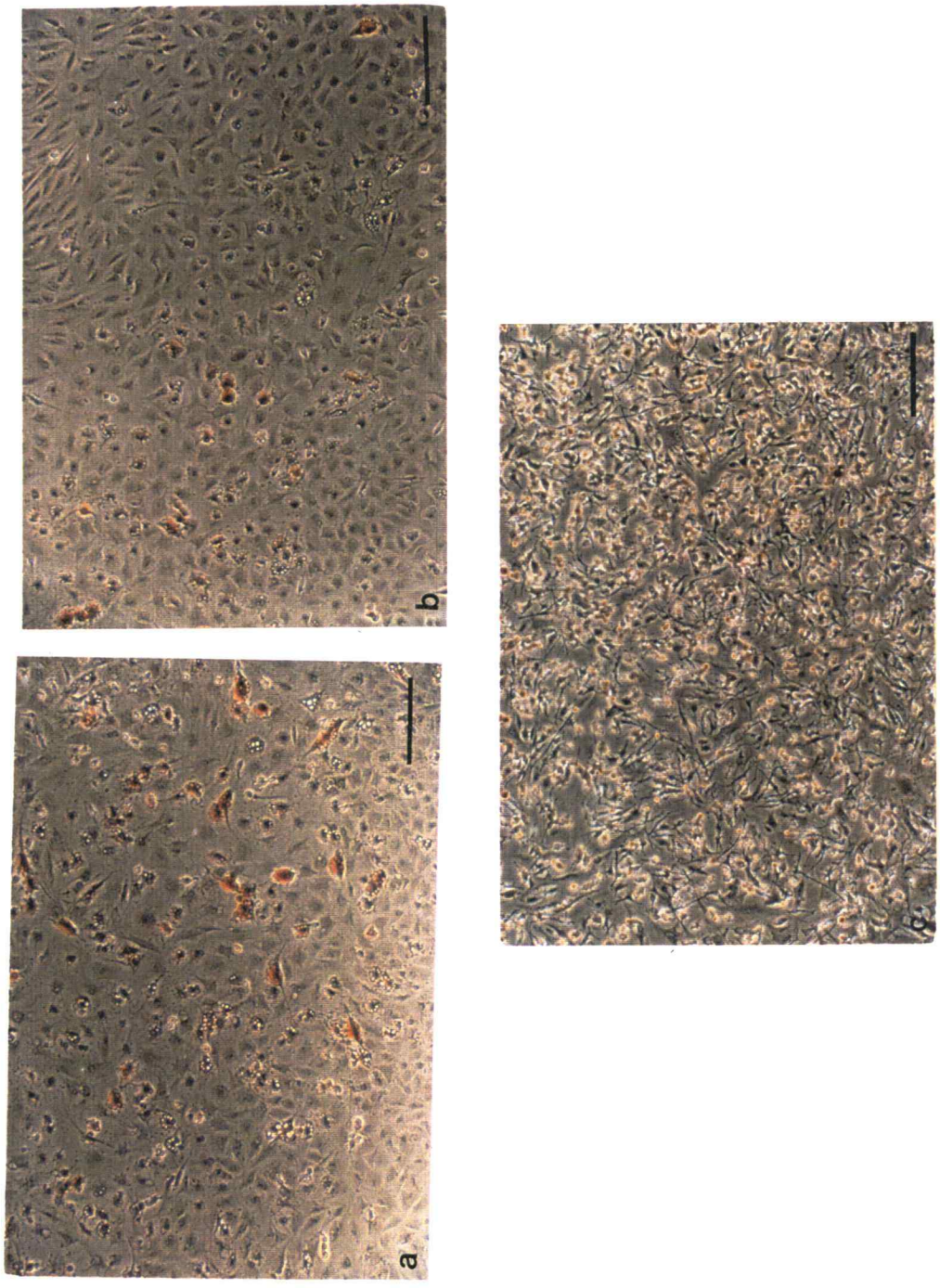


Figure 2.5

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# Chapter 3

## EFFECT OF AGE ON THE DIFFERENTIATION OF PORCINE ADIPOSE STROMAL-VASCULAR CELLS IN CULTURE

## ABSTRACT

Stromal-vascular (S-V) cells isolated from adipose tissue of newborn and mature pigs by collagenase digestion were used to evaluate differences in preadipocyte development. Cells were seeded at a density of $3 \times 10^4$ cells/cm$^2$ on 6 well (35 mm) tissue culture plates in 3 ml DME/HAM's medium plus 10% fetal calf serum and cultured at 37 °C under a humidified atmosphere of 95% air:5% $CO_2$ for 24 h. Cells were then washed thoroughly in DME/HAM's medium without fetal calf serum and maintained in serum free (SF) medium or SF medium supplemented with 2.5% newborn pig serum (NBPS) or mature pig serum (MPS) for 12 days. Newborn pig cells cultured in SF medium had higher sn-glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) specific activity than mature pig cells cultured in the same medium. Neither NBPS or MPS affected GPDH specific activity of newborn pig cell cultures. Expressed on per unit DNA basis, GPDH activity of mature pig cells cultured in SF medium was higher than that of newborn pig cells, reflecting the larger mature pig cell size. No differences were observed between GPDH specific activity in mature pig cells cultured in NBPS or MPS. GPDH specific activity was higher ($P<.05$), as was DNA concentration per well ($P<.05$), in newborn pig cells with either serum supplementation. Protein per well was not significantly different in cultures of newborn versus mature pig cells cultured in the same medium. Protein:DNA ratios were higher ($P<.05$) in cultures of mature pig cells. Cells from newborn pigs appear to replicate faster and to be more responsive to serum borne factors influencing S-V cell growth and development in culture. This

study demonstrates that intrinsic activity of the cells rather than serum borne factors are responsible for the divergence in the pattern of adipose tissue cellularity of neonatal and postneonatal pigs.

## INTRODUCTION

Age related changes in body composition have been considered unavoidable in the animal. Several structural changes take place in middle and late adulthood in the human. The lean body mass decreases and adipose tissue mass increases (Rudman, 1985). Several age related changes in adipose tissue mass and adipocyte function have been observed in rats (Kirkland and Dax, 1984). Mechanisms controlling age related changes in adipose tissue mass have not been fully elucidated.

In vivo studies have shown that adipose tissue contains cell fractions which are able to proliferate and differentiate into mature adipocytes (Hollenberg and Vost, 1968; Greenwood and Hirsch, 1974; Gaben-Cogneville and Swerczewski 1979). Primary cultures of stromal-vascular cells (S-V) isolated from adipose tissue of various species including man have shown the existence of cells capable of proliferating and acquiring adipocyte phenotype and biochemical characteristics (Bjorntorp et al.,1978; Hauner et al.,1989; Ramsay et al., 1989a). The growth and development of S-V cells in culture provide a valid model of adipocyte development in vivo (Hauner and Loffler, 1987). Djian et al. (1983) and Kirkland et al. (1990) have used cell culture technique to explore the cellular basis responsible for some of the changes in adipose tissue mass and function during aging. Adipocyte precursor cells from young animals may replicate faster than cells from the older animals (Djian et al., 1983; Kirkland et al., 1990) and young animals contain more clones capable of full differentiation into adipocytes (Kirkland et al., 1990). Hauner

et al. (1989) concluded that adipose tissue obtained from an animal at any age contains a pool of preadipocytes and decreased ability of cells to differentiate as animal gets older is due to inability of precursor cells to respond to extracellular stimuli and or decreased number of precursor clones.

Age dependent changes in the levels of serum hormones may have possible regulatory effect on preadipocyte growth and development. The fetal levels of insulin were very low in comparison to postnatal levels in pigs (Martin et al., 1985). The concentrations of growth hormone in the serum of fetal pigs have been shown to be higher than the concentrations in postnatal pig serum (Martin et al., 1984; 1985). In rodents, levels of circulating insulin-like growth factor-1 (IGF-1) were lower in fetal than postnatal mice (D'Ercole and Underwood, 1980). Another possible regulating hormone that could be affected by age is triiodothyronine ($T_3$). $T_3$ is three times higher in postnatal pigs than in fetal pig serum (Martin et al. 1984). Cortisol concentration is higher in fetal and neonatal pigs and decreases with maturity (Dvorak, 1972). Changes in serum borne components or factors influence the growth and development of preadipocytes (Ramsay et al., 1987a). The effect of aging on the ability of S-V cells to proliferate and differentiate and the capability of sera obtained from pigs of differing ages to promote proliferation and differentiation have not been studied in primary cultures of porcine S-V cells. Therefore, the purpose of this study was to determine whether age related physiological factors regulate serum adipogenic components and differences in sensitivity and responsiveness of cells to these adipogenic components.

## MATERIALS

Dulbecco's Modified Eagle's Medium (DME, D-5523), Nutrient mixture F-12 (HAM, N-6760), dihydroxy acetone phosphate (DHAP, D-7137), reduced nicotinamide adenine dinucleotide (NADH, N-8219) gentamicin sulfate (G-1264), hydrocortisone (H-0135), insulin (I-1882), triiodothyronine (T-5516), bovine transferrin (T-8027), hematoxylin (HHS-2-16), were purchased from Sigma Chemical Co.(St. Louis, MO). Bovine serum albumin (BSA, Bovuminar Reagent CRG-7) was purchased from Armour Pharmaceutical Co. (Tarrytown,NY); collagenase (type I) from Worthington Biochemical (Freehold, N.J.); thiamylal-sodium (Biotal) from Boehringer-Inglheim Animal Health Inc.(St Joseph, MO); fetal calf serum (FCS) from Intergen Co.. (Purchase, N Y); Fungizone from Gibco BRL (Gaithersburg, MD); and Prepodyne from AMSCO, Medical Products Division (Erie, PA). All other reagents were of analytical grade.

## METHODS

### Animal and biopsy procedure

Crossbred pigs less than one day old from a commercial producer were killed by $CO_2$ asphyxiation. Pigs were scrubbed with Prepodyne and rinsed thoroughly with 70% ethanol solution, and placed on a sterile surgical tray in a laminar flow hood. An incision was made with a sterile scalpel through the skin from about .5 cm posterior to the base of the skull along the sagittal plane to the scapula and from the midline about 3 cm laterally on both ends. Adipose tissue in the exposed area was carefully removed from the underlying tissue with sterile forceps and scissors.

For experiments utilizing cells from mature pigs, adipose tissue samples from subcutaneous neck region were obtained from 7 month old crossbred barrows during slaughter as previously described by Akanbi et al. (1990).

Sera collection

Blood was collected by aortic puncture at time of slaughter from 7 month old (110 Kg) crossbred barrows that have been maintained on ad libitum corn-soybean based diet. Blood was collected from newborn pigs by venipuncture of the anterior vena cava. Blood samples were allowed to clot overnight at 4 °C. After clotting, the sera were separated by centrifugation and filter sterilized using a 0.45 $\mu$m sterile acrodisc low protein binding filter (Gelman Sciences, Ann Arbor, MI). Sera from six pigs were pooled before use in culture.

Stromal-Vascular Cells Isolation

Dissected tissue samples were put in a petri dish containing Krebs-Ringer bicarbonate buffer (KRB, 37 °C; pH 7.4) 118 mM NaCl, 4.8 mM KCl, 10 mM HEPES, 5 mM glucose and 40 mg/L gentamicin sulfate, equilibrated with 95 % O2 : 5 %$CO_2$ and sterilized by filtering through a 0.22 $\mu$m acrodisc filter (Nalgene Co, Rochester, NY). Adipose tissue samples (3 g) were minced with a pair of sterile scissors and digested in a 25 ml digestion flask at 37 °C in a gyratory water bath for 1 hr with 9 ml filter sterilized KRB buffer containing 3 % BSA and 2 mg/ml collagenase. Digested tissue was filtered through a sterile single layer of polyester chiffon into 50 ml sterile polypropylene tubes. Floating adipocytes were separated

from other cells by aspirating the infranatant with a sterile syringe fitted with a long needle. The infranatant cell suspension was centrifuged at 800 x g for 10 min. S-V pellets were washed three times in DME/HAM medium (1:1 v/v) containing 15 mM NaHCO3, 15 mM HEPES buffer (pH 7.4), 40 mg/L gentamicin sulfate and 2 mg/L Fungizone and supplemented with 10% FCS (plating medium).

Cell Culture

Aliquots of the S-V cells were removed, stained with Rappaport's stain and counted on a hemocytometer. S-V cells were seeded in 3 ml plating medium on Corning 6 well (35 mm) tissue culture plates at a density of $3 \times 10^4$ cells/cm$^2$. Cells were cultured at 37 °C under a humidified atmosphere of 95% air: 5% CO; 24 hours later cells were washed 2x 5 minutes and 1x 1 hour with plating medium without FCS. Cells were subsequently maintained in test media. Test media consisted of 20 $\mu$U/ml insulin, 1 ng/ml triiodothyronine, 25 ng/ ml hydrocortisone, 10 $\mu$g/ml transferrin and 2.5% treatment sera as indicated in figures and legends. Test media were changed every 3 days until day 12 when cultures were terminated and protein, DNA and sn-glycerol-3-phosphate dehydrogenase were determined.

Enzyme Analysis

Sn-glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) was measured by a spectrophotometric methods for determination of oxidized NADH during GPDH-catalyzed reduction of DHAP (Kozak and Jensen, 1974) as modified by Wise and Green, (1979).

DNA and Protein Content

DNA was assayed as described by LaBarca and Paigen (1980) using salmon testes DNA as a standard. Protein content was determined by bicinchoninic acid (BCA) method using bovine serum albumin as a standard (Pierce Chemical CO., Rockford, IL).

Histochemistry

Representative wells from each treatment were fixed in 10% formalin, stained with oil red O for lipid and counterstained with Harris hematoxylin (Boon and Drijver, 1986) after 12 d of exposure to test media.

Statistical Analysis

This experiment used 2 x 3 factorial arrangement of treatments in a complete randomized design (Steel and Torrie, 1980). Data were subjected to analysis of variance and contrast between means were determined (SAS, 1987).

## RESULTS

Stromal-vascular cells obtained from adipose tissue from subcutaneous neck region of newborn pigs (NBPC) and mature pigs (MPC) were used to compare age dependent differences in growth and development of adipocyte precursor cells cultured in either newborn pig serum (NBPS) or mature pig serum (MPS). Cells were allowed to adhere for 24 h in DME/HAM's medium supplemented with 10% FCS. Unattached cells, mainly red blood cells and tissue debris were removed by

extensive washing with DME/HAM's medium. The adherent cells morphologically have the resemblance of fibroblast. Figure 3.1 shows the photomicrograph of NBPC and MPC maintained in DME/HAM's medium or DME/HAM's medium supplemented with hormones (serum free) for 12 d. NBPC had very good adherence to the surface of the culture dish. Some MPC were so loosely attached to the culture dish that by d 12 in DME/HAM's medium most of the cells detached and floated away leaving few or no attached cells in some culture dishes. Lipid histochemistry revealed a higher number of lipid filled cells in NBPC cultured in serum free medium compared with MPC cultured in the same medium (Fig. 3.1). Figure 3.2 shows the photomicrographs of NBPC and MPC cultured in either NBPS or MPS. There are more nuclei in cultures of NBPC in either NBPS or MPS than MPC in the same medium. The photomicrographs also reveal that NBPC cultured in either NBPS or MPS contained more numerous oil red O stained cells than MPC in the same media.

Figure 3.3 shows the cellular protein content in cultured cells. There was a significant difference between cellular protein content of NBPC and MPC cultured in serum free medium ($P<.05$) and between cellular protein content of NBPC and MPC cultured in NBPS ($P<.05$). Protein content of NBPC and MPC cultured in MPS did not differ ($P>.05$).

The DNA content of cultured cells is shown in Figure 3.4. There was higher DNA content ($P<.05$) in NBPC cultured in serum free medium compared with MPC cultured in the same medium. NBPC cultured in NBPS had greater ($P<.05$) DNA content than MPC cultured in the same medium and there was higher ($P<.05$) DNA in cultures of NBPC in MPS than in cultures of MPC in the same medium. Figure

3.5 shows the protein:DNA ratio of cultured cells. MPC had higher protein:DNA ratio than NBPC cultured in the same medium.

Differentiation of S-V cells was assessed by determination of sn-glycerol-3-phosphate dehydrogenase activity on day 12 of culture and the morphological criterion of differentiation was the presence of lipid droplets in the cytoplasm. Figure 3.6 shows GPDH activity in cultured cells. Culture media have profound effect on the differentiation of S-V cells. Cells grown in serum free medium (DME/HAM supplemented with 20 $\mu$m/ml insulin, 25 ng/ml hydrocortisone, 1 ng/ml triiodothyronine and 10 $\mu$g ml transferrin) had higher GPDH specific activity than cells grown in medium supplemented with 2.5% NBPS or MPS. GPDH specific activity was higher ($P<.05$) in NBPC cultured in serum free medium, NBPS or MPS than it was in MPC cultured in the same media. When the enzyme activity was expressed on per DNA basis, MPC cultured in serum free medium had the highest GPDH activity ($P<.05$) compared to every other culture, and NBPC cultured in serum free media had higher GPDH activity per DNA than NBPC cultured in NBPS or MPS. There was no difference between GPDH activity per DNA in NBPC cultured in NBPS and MPS. GPDH per well was higher ($P<.05$) in cultures of NBPC than cultures of MPC in the same medium.

## DISCUSSION

In the present study S-V cells obtained from two age groups of pigs have been used to evaluate the effects of age on the capacities of S-V cells to grow and differentiate in primary cultures. The ability of sera obtained from these two groups of pig to promote growth and development of S-V cells in culture was also

examined. Determination of GPDH activity in culture wells was used to assess differentiation (Pairault and Green, 1979; Wiederer and Loffler, 1987; Hauner et al., 1989; Gaben-Cogneville et al., 1990). Appearance of cytoplasmic lipid droplets was also used as morphological criterion of S-V cell differentiation.

S-V cells from the two groups of pigs were seeded at the same density in DME/HAM's medium at the beginning of the culture period but at the time the cultures were terminated there were fewer cells left in cultures of MPC as compared with NBPC (Fig. 3.1). This phenomenon was not due to cell detachment as a result of cytoplasmic lipid deposition which might make lipid filled cells become buoyant. There were no lipid droplets observed in cells maintained in DME/HAM's throughout the culture period. The disparities in the ability of NBPC and MPC to stay attached in culture might be due to differential regulation of genes. It is possible that certain factors are secreted by S-V cells that facilitate cell attachment and because of "ageing" the expression of genes responsible for such factors are diminished. However, supplementation of DME/HAM's medium with hormones might have stimulated the expression of these genes in "aged cells" since more cells are visible in cultures of MPC in hormone supplemented medium as compared to DME/HAM's medium alone. Until the different factors secreted by S-V cells are characterized, their roles in cell attachment defined and effect of age on the secretions determined, the reasons why S-V cells obtained from young animals attached better in medium devoid of hormones or growth factors remain a mere speculation.

Under the conditions employed in this study age caused differential responsiveness of S-V cells to hormones and growth factors in the culture media

hence the differences in differentiation activities between NBPC and MPC. It is not known whether there are differences in receptors and postreceptor activities in NBPC and MPC. The differences in receptor and postreceptor events between the two cell types might influence the expression and activity of enzymes and growth regulators that might be responsible in differentiation events (Djian et al, 1983).

Endocrine substances can influence adipose tissue accretion (Weekes, 1983; Muir, 1985; Tucker and Merkel, 1987). Most of the evidence regarding endocrine influence on adipocyte development come from studies of cells in vitro. In vitro approach permits clear interpretation of the effect of a single endocrine substance on a single cell type. The results of the present study cannot be solely attributed to endocrine status of young animals. Level of insulin is lower in fetal than postnatal pig serum (Martin et al., 1985). Insulin did not promote differentiation in primary cultures of pig S-V cells (Hentges and Hausman, 1989). Addition of insulin to fetal pig serum which is inherently low in insulin enhanced differentiation activity in primary cultures of rat S-V cells (Hausman and Jewell, 1988). The disparities in these results might be due to differences in species of animal from which the S-V cells were obtained and the complex interactions between hormones and growth factors in serum. Fetal pigs have higher serum growth hormone levels when compared to postnatal pig serum (Martin et al., 1984; 1985). Growth hormone has only been shown to promote adipogenic activity in adipocyte-like cell lines (Morikawa et al., 1982; 1984; Doglio et al., 1986). Growth hormone either had no effect on differentiation activity (Wiederer and Loffler, 1987; Kalbitz and Mueller, 1990) or decreased differentiation in primary cultures (Hausman and Martin, 1989).

IGF-1 (D'Ercole and Underwood, 1980) and $T_3$ (Martin et al., 1984) are lower in fetal and postnatal animals. Thyroid hormone level influenced adipocyte number in vivo (Picon and Levacher, 1979) and adipocyte development in vitro (Ramsay, 1985). IGF-1 can induce differentiation in primary cultures of pig S-V cells (Ramsay et al., 1989a). In theory, therefore, endocrine status of young pigs does not support adipocyte development. The hormonal millieu alone could not explain the higher differentiation activity observed in cells cultured with NBPS. The present study has demonstrated that serum borne factors have a major influence on the growth and development of preadipocytes and the activity of these factor(s) can be influenced by age of the animal. Sypniewska (1989) found that differentiation activity in cultured rat S-V cells declined with age of plasma donors and suggested that there are factors in plasma other than hormone that could influence preadipocyte differentiation. The results of present study support the view of Sypniewska (1989). However, the degree of differentiation in the present study does not agree with the work of Ramsay et al. (1987b) who reported that fetal sera did not promote adipocyte formation. The discrepancies might be due to differences in the culture conditions and differences in adipose stromal-vascular cells used. Ramsay et al. (1987b) used stromal-vascular cells obtained from adipose tissue of rats and did not use hormone supplemented medium for basal medium. The higher differentiation activity in NBPS in the present study was however, not potentiated by the addition of insulin, hydrocortisone and $T_3$ to the basal DME/HAM's medium since GPDH activity in MPC cultured in NBPS was significantly different from GPDH activity in MPS (Fig. 3.3).

The DNA data (Fig. 3.4) supports the observation that nuclei in NBPC are more numerous than nuclei in MPC cultures (Fig. 3.2), implying that NBPC may undergo more replication than MPC and as a result there are more cells and clones that could undergo replication and differentiation in NBPC. The present data also indicate that there is a decline in cellular replicative capacity in pig S-V cells as animal grows older. This agrees with observations in other systems (Djian et al. 1983; Kirkland et al.,1990; Smith et al.,1978; Didinsky and Rheinwald, 1981; Hayflick, 1965; Martin et al., 1970; Raes and Remacle, 1983; Rheinwald and Green, 1977; Schultz and Lipton, 1982). Lumpkin et al (1986) found a high number of anti-proliferative mRNAs in aging human diploid fibroblasts and concluded that decline in cellular replicative capacity with aging may be associated with the production of inhibitors of replication. Evidence shows that there are periods during growth that the rate of cell replication diminishes during adipose tissue growth and the subsequent growth is by increase in cell size. Measurement of adipocyte number in pigs during growth showed that cell number continues to increase until 5 or 6 weeks of age (Gurr et al., 1977). Severe feed restriction to suckling pigs did not change the adipocyte number in subcutaneous depot (Lee et al., 1973a,b) indicating that in the fetal or early postnatal period of growth adipocyte precursor cells proliferate and establish a precursor pool that continues to increase in size. This does not mean however, that proliferation of precursor cells in the stromal fraction of adipose tissue is totally ceased in mature animal. Preadipocytes in postnatal pig adipose stromal fraction can proliferate and differentiate in culture (Novakofski, 1981). The decline in replicative capacity of MPC in vitro corresponds

to that of in vivo adipose tissue development during postnatal period of life since GPDH activity per cell was highest in MPC cultured in serum free medium. The high GPDH activity per cell may be attributable to increase in cell size (protein:DNA ratio) in MPC. Because of availability of space for cells to spread and grow MPC were able to attain larger size than NBPC. Lack of available space in culture wells due to crowding as a result of increase cell number might have contributed to the smaller cell size in cultures of NBPC. Cellular differentiation and hypertrophy appear to occur more readily than does replication in adipose tissue of postnatal pigs during growth (Anderson and Kauffman 1973; Mersmann et al., 1975; Hood and Allen, 1977).

Fetal sera promotes rapid cellular replication in culture. Ramsay et al. (1987b) reported that fetal pig sera promoted higher incorporation of $^{3}$H-thymidine into cultured cells than postnatal pig sera. The higher DNA levels in cultures grown in NBPS indicates that NBPS supported more cell proliferation than MPS. This might be due to the presence of higher levels and/or activity of mitogenic factors in NBPS. Although, there is no difference between DNA levels in NBPS and MPS using MPC, the lack of difference might be due to decreased responsiveness of MPC to mitogenic factors present in serum and/or decreased replicative capacity of the cells. The mechanisms responsible for differences in the ability of S-V cells to multiply and differentiate due to age are yet to be elucidated. Djian et al. (1983) and Kirkland et al. (1990) however suggested that factors such as gene regulation, hormones, autocrine and paracrine factors which may mediate the effect of aging could affect replicative and differentiation rate of S-V cells and must be considered

as possible mechanisms responsible for the differences.

The fact that age could affect development of preadipocytes and could change the activity and/or levels of adipogenic and growth factors in serum is intriguing and requires further exploration. Identification of these factors could enhance our ability to manipulate the growth and composition of meat producing animals.

FIGURE 3.1. *Photomicrographs of pig adipose stromal-vascular cells grown in medium.* Cells were inoculated at a density of 3 x $10^4$ cells/$cm^2$ and grown in DME/HAM's medium containing 10% FCS for 24 h. after which cells were washed with DME/HAM's medium without FCS. Cells were subsequently grown in either DME/HAM's medium containing 10 $\mu$g/ml transferrin without hormone supplementation or with supplementation (20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine; SF). (A) newborn pig cells (NBPC) and (B) mature pig cells (MPC) grown in DME/HAM's medium without hormone supplementation. (C) NBPC and (D) MPC grown in SF. On day 12 of culture cells were washed with phosphate buffered saline, fixed in 10% formalin, stained with oil red O and hematoxylin. Cytoplasmic lipid droplets were stained red with oil red O while nuclei were stained blue with hematoxylin. There were no lipid droplets in cells maintained in DME/HAM's medium without hormone supplementation. Hormone supplementation stimulated lipid deposition. Note the abundant lipid containing in C as compared to D and fewer cells in B as compared to A. Magnification = 100x. Bar = 200 $\mu$.

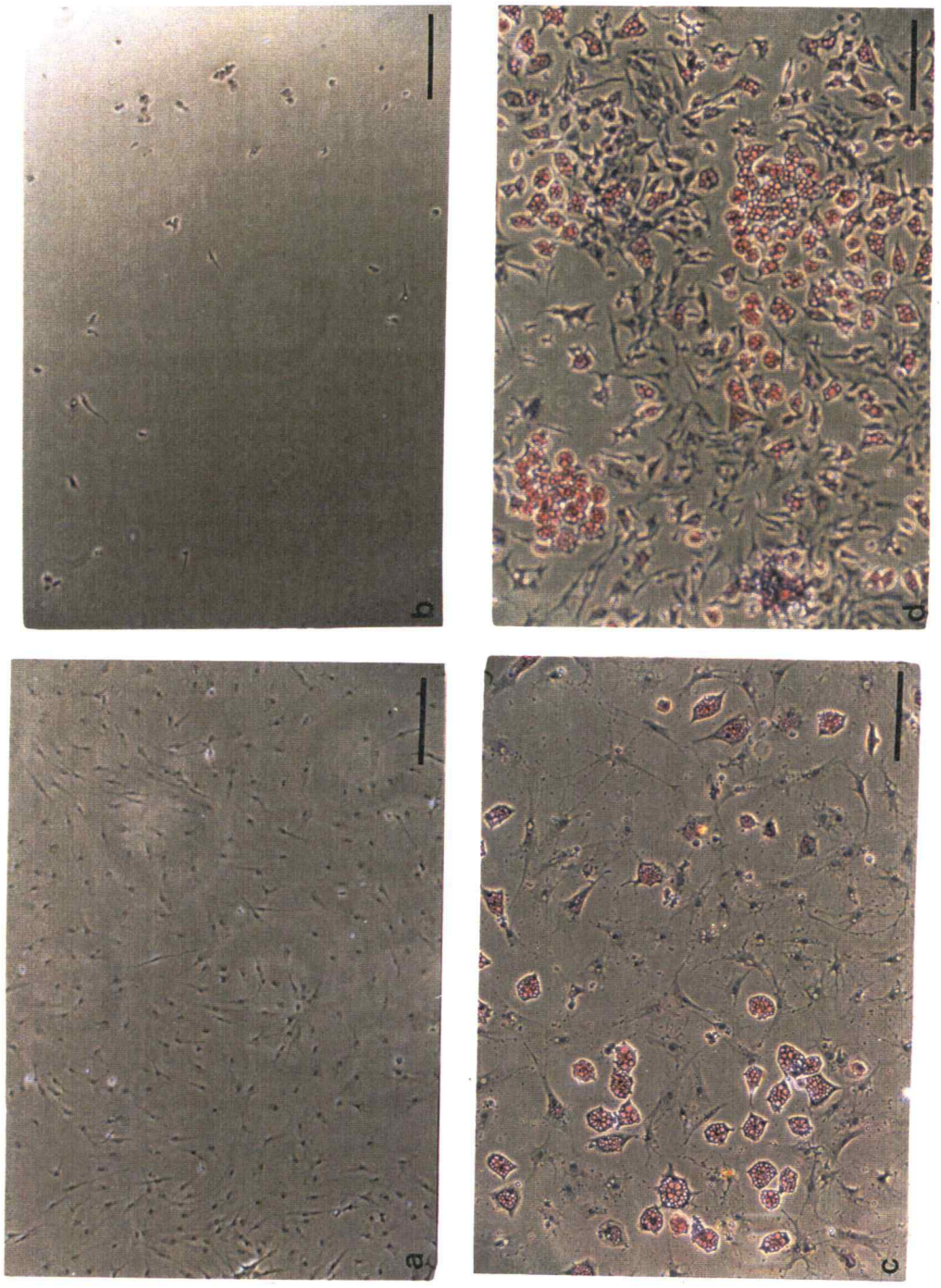


Figure 3.1

FIGURE 3.2. *Photomicrographs of adipose stromal-vascular cells obtained from newborn pigs (NBPC) and mature pigs (MPC) cultured in serum from pigs of either ages.* Cells were inoculated at a density of 3 x $10^4$ cells/$cm^2$ and maintained in DME/HAM's medium containing 10% FCS for 24 h. after which cells were washed in DME/HAM's medium without FCS. Cells were subsequently maintained in DME/HAM's medium containing 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% newborn pig (NBPS) or mature pig (MPS) serum for 12 d. (A) MPC in NBPS; (B) NBPC in NBPS; (C) MPC in MPS and (D) NBPC in MPS. On day 12 cells were washed with phosphate buffered saline and fixed in 10% formalin. Cells were then stained with oil red O and counterstained with hematoxylin and photomicrography was performed. Oil red O stained the cytoplasmic lipid droplets red and hematoxylin stained the cell nuclei blue. Cells stained with oil red O indicate lipid deposition. Note the more numerous nuclei in B compared to A and in D compared to C. There are more lipid containing cells in B compared to A and in D compared to C. Magnification = 100x. Bar = 200 $\mu$.

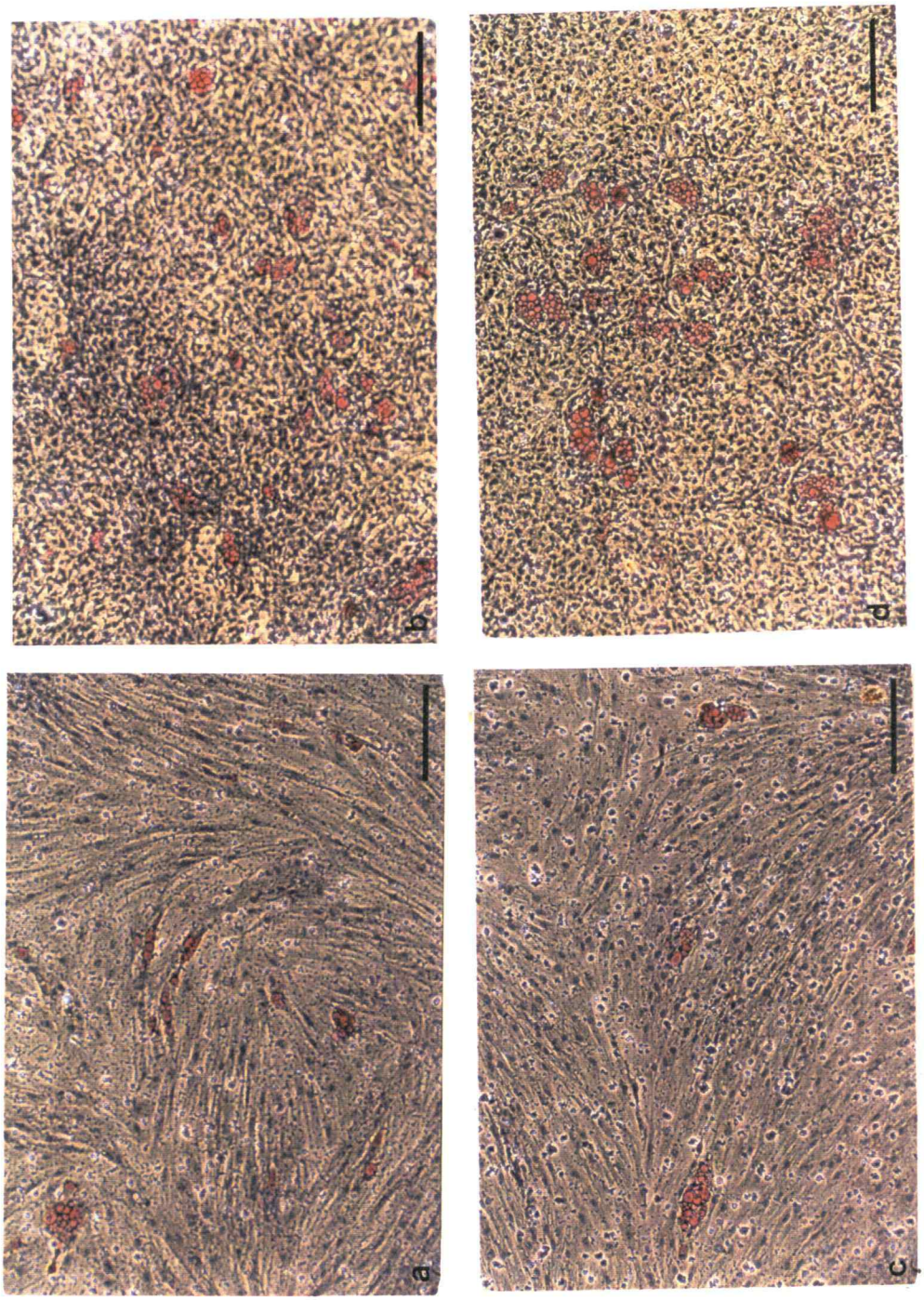


Figure 3.2

FIGURE 3.3. *Protein content of cultured porcine stromal-vascular cells obtained from adipose tissue of newborn and mature pigs.* Cells were seeded on 6 well plate at a density of 3 x $10^4$ cells/$cm^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine without serum (SF) or with 2.5% newborn pig serum (NBPS) or 2.5% mature pig serum (MPS). Cellular protein content in cultured cells was determined on d 12. Values are means ± SE of three independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P < .05$.

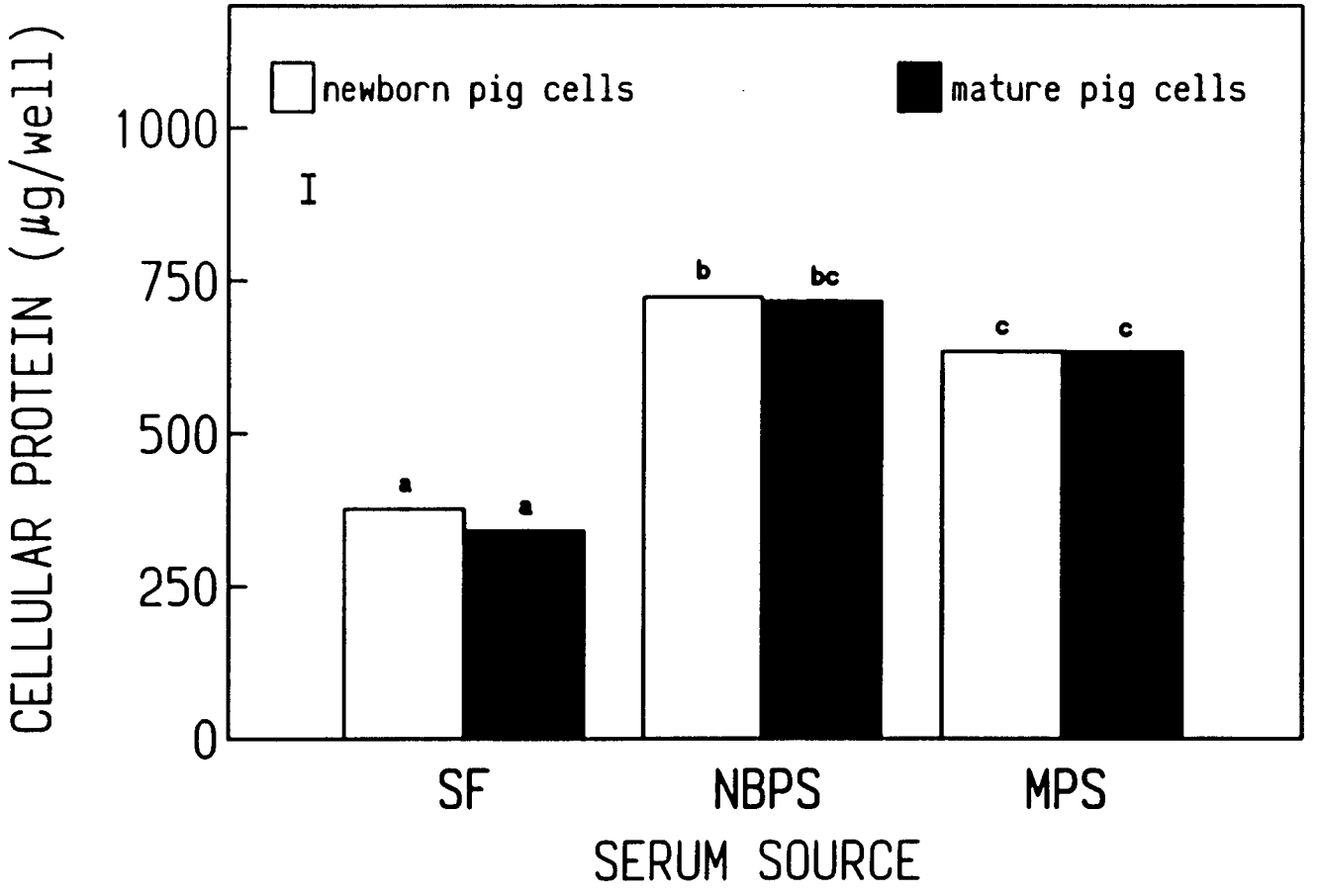
CELLULAR PROTEIN (µg/well)
1000
750
500
250
0
newborn pig cells
mature pig cells
I
a
a
b
bc
c
c
SF
NBPS
MPS
SERUM SOURCE

Figure 3.3

FIGURE 3.4. *DNA content of cultured porcine stromal-vascular cells obtained from adipose tissue of newborn and mature pigs.* Cells were seeded on 6 well plate at a density of $3 \times 10^4$ cells/cm$^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine without serum (SF) or with 2.5% newborn pig serum (NBPS) or 2.5% mature pig serum (MPS). DNA content in cultured cells was determined on d 12. Values are means ± SE of three independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P<.05$.

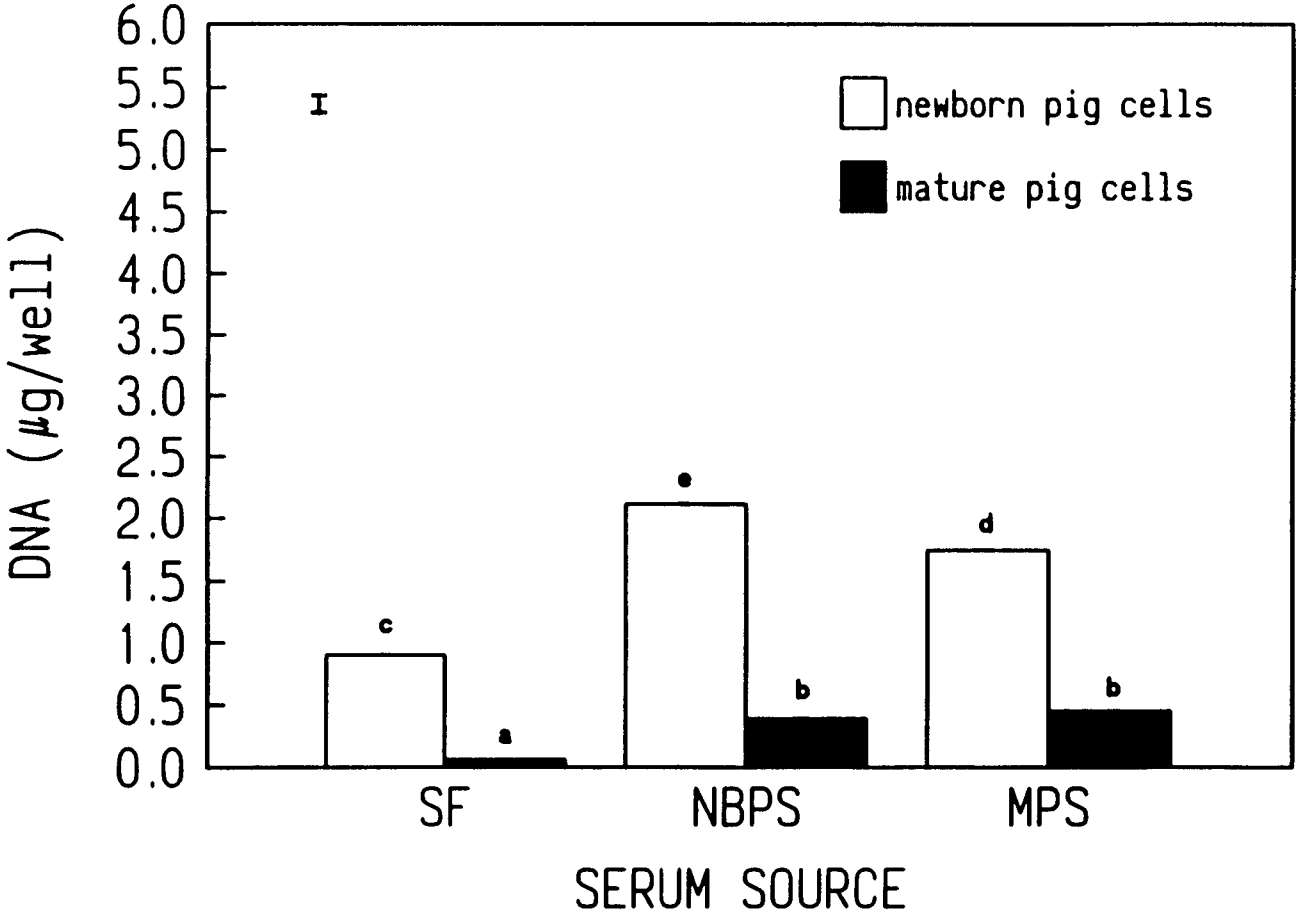
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
DNA (µg/well)
I
newborn pig cells
mature pig cells
c
a
e
b
d
b
SF
NBPS
MPS
SERUM SOURCE

Figure 3.4

FIGURE 3.5. *Cellular protein:DNA ratio of cultured porcine stromal-vascular cells obtained from adipose tissue of newborn and mature pigs.* Cells were seeded on 6 well plate at a density of 3 x $10^4$ cells/$cm^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine without serum (SF) or with 2.5% newborn pig serum (NBPS) or 2.5% mature pig serum (MPS). Protein and DNA contents in cultured cells were determined on d 12 and ratio of protein to DNA was calculated. Values are means ± SE of three independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P < .05$.

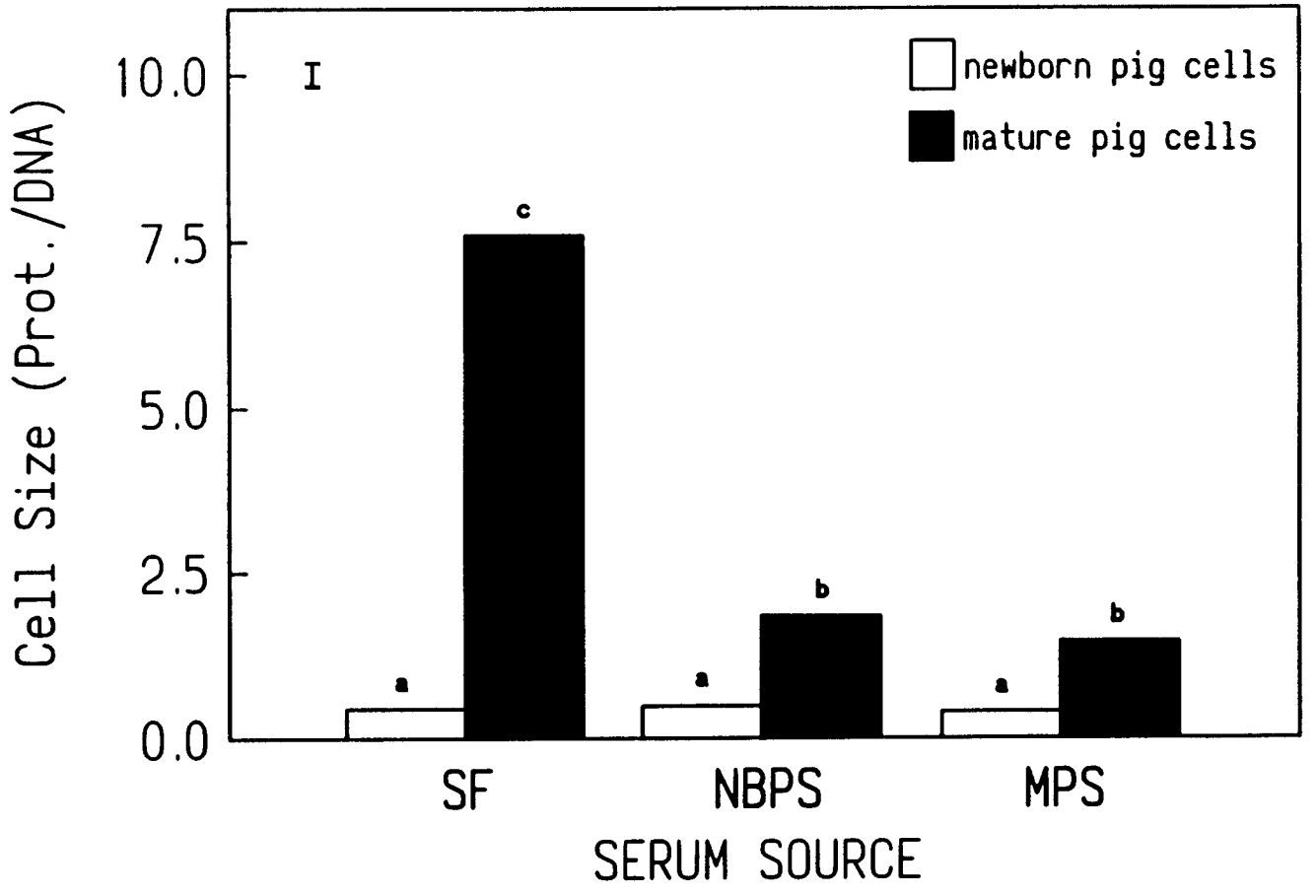
I
newborn pig cells
mature pig cells
Cell Size (Prot./DNA)
10.0
7.5
5.0
2.5
0.0
a
c
a
b
a
b
SF
NBPS
MPS
SERUM SOURCE

Figure 3.5

FIGURE 3.6. *Sn-glycerol-3-phosphate dehydrogenase in stromal-vascular cells obtained from adipose tissue of newborn and mature pigs.* Cells were inoculated on 6 well plate at a density of 3 x $10^4$ cells/$cm^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine without serum (SF) or with 2.5% newborn pig serum (NBPS) or 2.5% mature pig serum (MPS). GPDH activity in cultured cells was determined on d 12. (A) Enzyme activity expressed as per mg protein, (B) enzyme activity expressed on per well basis and (C) enzyme activity expressed on DNA basis. Values are means ± SE of three independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P<.05$.

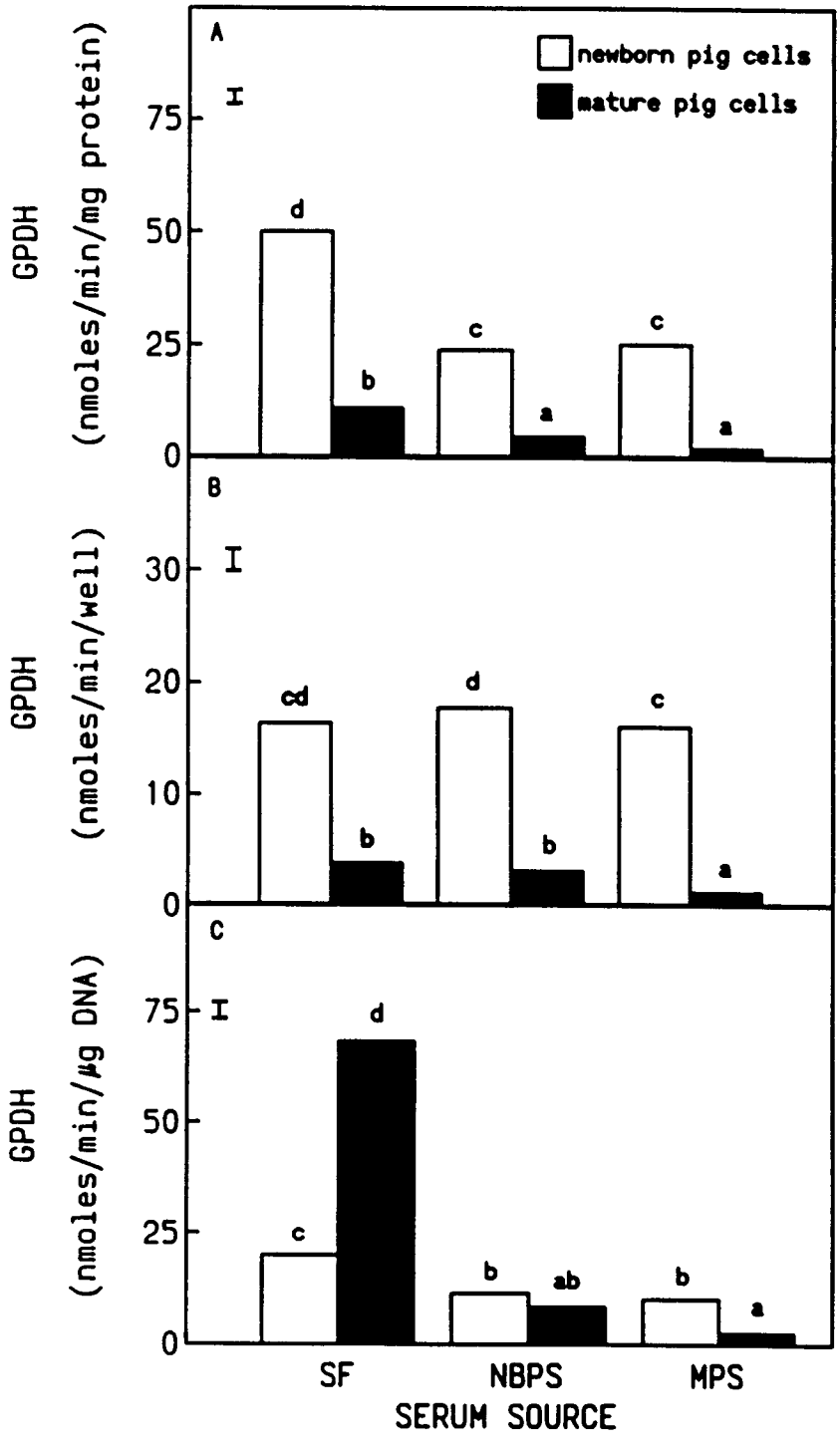
A
newborn pig cells
mature pig cells
GPDH (nmoles/min/mg protein)
75
50
25
0
d
b
c
a
c
a
B
GPDH (nmoles/min/well)
30
20
10
0
cd
b
d
b
c
a
C
GPDH (nmoles/min/µg DNA)
75
50
25
0
c
d
b
ab
b
a
SF
NBPS
MPS
SERUM SOURCE

Figure 3.6

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# Chapter 4

# EFFECT OF SERA FROM FED AND FASTED PIGS ON THE DIFFERENTIATION OF ADIPOSE STROMAL-VASCULAR CELLS IN CULTURE

## ABSTRACT

Effect of 3-5 days of fasting on the ability of serum to promote growth and development of preadipocytes in primary cultures of stromal-vascular cells from adipose tissue of pig and rat was studied. Sn-glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) specific activity was significantly increased ($P < .05$) in cultures of rat or pig adipose stromal-vascular cells grown in serum from pigs fasted for 3 or 5 days as compared to serum obtained from fed pigs. There was no difference ($P > .05$) between GPDH activity of rat or pig cells cultured in sera obtained from pigs fasted for 3 and 5 days. Protein and DNA contents of pig cell cultures showed no statistical differences ($P > .05$) between treatments. Fasted pig sera decreased ($P < .05$) DNA content of rat cell cultures but protein content was not affected ($P > .05$). These results suggest that a 3-5 day fast may induce changes in serum levels or activities of factors influencing preadipocyte differentiation in culture.

## INTRODUCTION

Overfeeding by oral gavage has been shown to increase body lipid in rats (Jewell et al.,1988; Harris and Martin, 1986). Feeding of high fat diet to lean and obese pigs resulted in more carcass adipose tissue deposition in both strains of pigs (Mersmann et al., 1984). Increase in body lipid as a result of overfeeding or feeding of high fat can result from at least two possible phenomena. First, it may simply be filling of existing differentiated adipocytes with lipid. Secondly, increase in cell number of adipocytes due to the stimulation of primary preadipocyte proliferation and their subsequent differentiation into adipocytes may account for the increased body lipid deposition. An increase in adipose cell number in mature rats can result in response to being fed a highly palatable high fat or high sugar diet (Faust et al., 1978; Faust and Miller, 1983). Undernutrition of rats followed by rehabilitation resulted in a rapid deposition of fat in the body (Harris, 1980). Chronic undernutrition impaired fat cell replication and rehabilitation from undernutrition caused increased cell replication in the subcutaneous fat depots (Kirtland and Harris, 1980)

In vivo studies have shown that adipose tissue contains cell fractions which are able to proliferate and differentiate into mature adipocytes during adipose tissue growth (Hollenberg and Vost, 1968; Greenwood and Hirsch, 1974; Gaben-Cogneville and Swierczewski, 1979). The growth and development of adipose stromal-vascular cells in culture provide a valuable model for adipocyte development in vivo (Hauner and Loffler, 1987) and have been used to evaluate factors regulating

the differentiation of precursor cells into adpocytes (Loffler and Hauner, 1987). Jewell et al. (1988) have evaluated the effect of sera obtained from control and over-fed rats on preadipocyte proliferation and differentiation in culture. Serum from over-fed rats increased preadipocyte differentiation but decreased proliferation when compared to the control. Plasma from rats fed high fat, high sugar diet promoted a higher degree of lipid filling than plasma from fasted rats in culture of rat adipose stromal-vascular cells (Bjorntorp et al., 1985). Changes in serum borne components or factors have a profound control on growth and development of preadipocytes (Ramsay et al., 1987). Little is known about the effects of fasting on serum factors that may influence the growth and development of preadipocytes in animals. The purpose of this study was to investigate fast-induced changes in the ability of serum to promote differentiation of pig and rat adipose stromal-vascular cells in culture.

## MATERIALS

Dulbecco's Modified Eagle's Medium (DME, D-5523), nutrient mixture F-12 (HAM, N-6760), dihydroxy acetone phosphate (DHAP, D-7137), reduced nicotinamide adenine dinucleotide (NADH, N-8219), gentamicin sulfate (G-1264), hydrocortisone (H-0135), insulin (I-1882), triiodothyronine (T-5516), bovine transferrin (T-8027), and hematoxylin (HHS-2-16) were purchased from Sigma Chemical Co.(St. Louis, MO). Bovine serum albumin (BSA, Bovuminar Reagent CRG-7) was purchased from Armour Pharmaceutical Co. (Tarrytown,NY);

collagenase (type I) from Worthington Biochemical (Freehold, N.J.); fetal calf serum (FCS) from Intergen Co.. (Purchase, N Y); Prepodyne from AMSCO, Medical Products Division (Erie, PA); and Fungizone from Gibco BRL (Gaithersburg, MD). All other reagents were of analytical grade.

## METHODS

### Animals

Eight crossbred barrows weighing between 90-100 Kg from Oregon State University Swine Center fed corn soybean meal based diet were randomly assigned to two treatment groups. The control group (fed) continued to be fed corn soybean based diet ad libitum. The other group was subjected to a five day fast with continuous access to water. Crossbred pigs less than one day old were obtained from a commercial producer and male Sprague-Dawley rats weighing 160-180 g were purchased from Simonsen Laboratories, Inc. (Gilroy, CA). Both newborn pigs and Sprague-Dawley rats served as cell donors.

### Sera Collection

Blood was collected from the fed and fasted pig groups on d 3 and d 5 of the experiment by venipuncture of the anterior vena cava using a 50 ml syringe fitted with long 14 gauge needle . Blood was allowed to clot overnight at 4 °C. After clotting, the sera were separated by centrifugation and filter sterilized using 0.45 $\mu$m sterile acrodisc, low protein binding filter (Gelman Sciences, Ann Arbor, MI). Pooled fed serum and pooled fasted pig serum were used in culture.

Stromal-vascular cell Isolation

Animals were killed by $CO_2$ asphyxiation, scrubbed with Prepodyne and rinsed with 70% ethanol solution. Disinfected pig or rat was placed on sterile surgical tray in a laminar flow hood. Incision was made in pig's skin from about .5 cm posterior to the base of the skull along the sagittal plane to the scapula and from the midline to about 3 cm laterally on both ends with a sterile scalpel. Adipose tissue sample in the exposed area was carefully removed from the underlying tissue with sterile forceps and scissors. For rats, incision was made at the ventral skin transversely at the median line just over the diaphragm with sterile scissors. The skin was cut longitudinally, grasping the skin on both sides of the cut with two pairs of sterile scissors the skin was pulled in the opposite directions to expose the ventral surface of the body. The inguinal fat pads on both thighs were removed from the underlying tissue with sterile forceps and scissors. Dissected tissue samples were put in separate petri dishes in a KRB solution containing 118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.3 mM $MgSO_4$, 10 mM HEPES, 5 mM glucose and 40 mg/L gentamicin sulfate, equilibrated with 95% $O_2$:5% $CO_2$ and sterilized by filtering through 0.45 $\mu$m Nalgene disposable syringe filter (Nalge Co., Rochester, NY). Adipose tissue samples (3 g) were minced with sterile scissors and digested at 37 °C in a gyratory water bath for 1 hr in a 25 ml polypropylene flask containing a filter sterilized solution of 18 mg collagenase in 9 ml of KRB buffer containing 3% BSA. Digested tissue was filtered through a sterile single layer of polyester chiffon, and cell suspension was centrifuged at 800 x g for 10 min. Stromal-vascular (S-V) pellets were washed three times in plating medium

(DME/Ham's medium; 1:1, v/v) containing 15 mM $NaHCO_3$, 15 mM HEPES buffer (pH 7.4), 40 mg/L gentamicin sulfate and 2 mg/l Fungizone and 10% FCS.

Cell Culture

Aliquots of the S-V cells were removed, stained with Rappaport's stain and counted on a hemocytometer. S-V cells were seeded in plating medium on Corning 6 well (35 mm) tissue culture plates at a density of 3 X $10^4$ cells/$cm^2$. Cells were cultured at 37 $^0$C under a humidified atmosphere of 95% air:5% $CO_2$; 24 hours later cells were washed 2 x 5 min. and 1 x 1 h. with plating medium without FCS to remove unattached materials, mainly erythrocytes and tissue debris. Cells were subsequently maintained in test media. Test media consisted of plating medium without FCS plus 20 $\mu$U/ml insulin, 1 ng/ml triiodothyronine, 10 $\mu$g/ml transferrin and 25 ng/ml hydrocortisone (ITTC) supplemented with 2.5% control pig serum, 3 d or 5 d fasted pig serum. Test media were changed every 3 days until day 12 when cultures were terminated and protein, DNA and sn-glycerol-3-phosphate dehydrogenase activity were determined.

Enzyme Analysis

Sn-glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) was measured by spectrophotometric methods of Kozak and Jensen (1974) as modified by Wise and Green (1979).

DNA and Protein Content

DNA was assayed as described by LaBarca and Paigen (1980) using salmon testes DNA as a standard. Protein content was determined by bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as a standard (Pierce Chemical Co., Rockford, IL).

Histochemistry

Representative wells from each treatment were fixed in 10% formalin, stained with oil red O for lipid and counterstained with Harris hematoxylin (Boon and Drijver, 1986) after 12 d of exposure to test media.

Statistical Analysis

Data were subjected to one-way analysis of variance according to General Linear Models Procedure and means separated by Least Significant Difference test (SAS, 1987).

## RESULTS

The influence of serum type on the cellular protein content of porcine S-V cell cultures is shown in Figure 4.1. There was no difference in the amount of protein in S-V cell cultures fed the different sera. Cellular protein levels in cultured rat S-V cells was not significantly affected by the different sera (Figure 4.2; $P > .05$). The capability of serum to stimulate porcine S-V cell replication as measured with DNA level in cultured cells was not influenced by fasting. S-V cell

DNA levels were not different in the three sera ($P > .05$; Figure 4.3), but there was a significant decrease ($P < .05$) in DNA level of rat S-V cells cultured in either fasted pig sera (Figure 4.4).

GPDH activity in porcine S-V cells as influenced by sera is shown in Figure 4.5. There was higher GPDH activity in cultures grown in serum obtained from fasted pigs than in cultures grown in serum obtained from fed pigs. There was no difference in GPDH activity of porcine S-V cells grown in serum obtained from 3 d or 5 d fasted pigs. The activity of GPDH in rat S-V cells as influenced by serum type is shown in Figure 4.6. GPDH activity was greater ($P < .05$) in cultures of rat S-V cells receiving either fasted pig serum than in cultures receiving fed pig serum from fed pigs. Sera from fasted pigs increased GPDH activity by over 90%. There was no difference in the ability of sera obtained from 3 and 5 day fasted pigs to stimulate GPDH activity in rat S-V cells. Porcine S-V cells with cytoplasmic lipid droplets appeared as big cluster of cells in cultures grown in either of the fasted pig sera (Figure 4.7). Oil red O staining revealed that there are more numerous cells with cytoplasmic lipid droplets in rat adipose S-V cells grown in fasted pig serum than in cells grown in fed pig serum (data not shown).

## DISCUSSION

One of the functions performed by adipocytes is the storage of energy as triglyceride during abundance of food supply and the release of the stored energy during deprivation. Studies of adipose tissue development showed that high fat feeding or overfeeding can result in increased adipocyte number in rats (Faust,

1978; Faust and Miller, 1983) consequently, resulting in increase in body lipid deposition (Jewell et al. 1988; Harris and Martin, 1986; Mersmann et al.,1984). At the cellular level little is known about the possible effect of fasting on preadipocyte development, except that adipocytes release their fat stores (Miller et al., 1983). Cell culture systems provide us with a tool to examine the effect of changes in serum borne factors on a particular tissue or cell type. This study used S-V cells from adipose tissue of pigs and rats to evaluate fast-induced changes in the ability of serum to support S-V cell growth and development. The results indicate that sera obtained from fasted pigs stimulated higher differentiation activity and lipid filling in both rat and pig adipose S-V cells in culture. This is in contrast to the work of Bjorntorp et al. (1985), who reported that there was little or no lipid filling in cells cultured with plasma from fasted rats; however, the present results are in agreement with another observation of Bjorntorp et al. (1985) that plasma from fasted rats supported GPDH activity.

Fasting is used primarily in the treatment of morbidly obese individuals to cause weight loss (Bloom,1959; Duncan et al.,1963). During nutritional deprivation the major metabolic fuel is from adipose tissue. Facilitatory action or permissive effects of a wide variety of hormones augment lipolytic activity in adipose tissue causing circulating free fatty acids and glycerol to rise. Blood serves as the vehicle for transporting all nutrients to individual cells; alterations in nutrient levels in the sera of fasted pigs could partly explain the present result. Increases in circulating lipids in fasted pig sera could enhance higher lipid deposition compared with fed pig serum and as a result there could be stimulation of GPDH activity to furnish the

glycerol backbone for triacylglycerol synthesis. Arachidonic acid has been shown to promote terminal differentiation and triacylglycerol accumulation in Ob17 cells (Gaillard et al. 1989). Long chain fatty acids have been shown to potentiate the effect of glucocorticoid (Abumrad et al., 1991). Abumrad et al. (1991) concluded that increase in availability of fatty acids might add to or modulate the induction of proteins necessary for preadipocyte differentiation. Therefore, rise in circulating fatty acids associated with fasting could be one of the reasons why sera obtained from fasted pigs stimulated higher differentiation activity in cultured S-V cells.

Changes in the concentrations of circulating hormones and blood metabolites have been reported during feed restriction in pigs (Kasser et al.,1981; Kornegay et al., 1964; Hodate et al.,1983; Buonomo and Baile, 1991). Hormones and growth factors present in the extracellular environment have profound influence on growth and function of cells (Darmon et al.,1981) and changes in serum borne components or factors have significant effect on growth and development of preadipocytes (Ramsay et al.,1987). Growth hormone increased the expression of differentiation-dependent mRNAs in Ob17 cells (Doglio et al., 1986) and stimulated differentiation and adipose conversion of 3T3 cells (Morikawa et al., 1982; Morikawa et al., 1984) and Ob17 cells (Ailhaud et al.,1983). Growth hormone had no effect on preadipocyte differentiation in rat (Wiederer and Loffler, 1987) and pig (Kalbitz and Mueller, 1990) and inhibited differentiation in pig primary cultures (Hausman and Martin, 1989). Growth hormone has been shown to be higher in plasma of fasted pigs (Kasser et al., 1981; Hodate et al., 1983; Buonomo and Baile, 1991). It is however unlikely that growth hormone differences can explain the increased GPDH

activity in cell cultures fed sera obtained from fasted pigs since growth hormone does not influence differentiation in primary cultures (Wiederer and Loffler, 1987; Hausman and Martin, 1989; Kalbitz and Mueller, 1990). Another possible regulating hormone in this system is cortisol. Feed restriction disrupted the circadian rhythm of plasma cortisol in pigs (Becker, 1990), and there was inverse relationship between feed intake and plasma concentrations of cortisol (Lepine et al.,1989). Increase in the level of plasma cortisol has also been observed in humans during fasting (Boyle et al.,1989). Glucocorticoids stimulated preadipocyte differentiation in culture (Chapman et al.,1985; Schiwek and Loffler, 1987) and have been found to be a major component of adipogenic activity in human serum (Schiwek and Loffler, 1987). Some of the variations in the ability of sera obtained from fed and fasted pigs may be attributable to variation in cortisol levels. However, the basal medium used in the present system consisted of insulin, hydrocortisone, triiodothyronine and transferrin. It is unlikely that the effect of fasted pig serum, which has been shown to be inherently lower in insulin than serum obtained from fed pigs (Buonomo and Baile, 1991), was masked by the addition of insulin or other hormones to the basal medium since GPDH activity, protein and DNA contents of cells cultured in basal medium alone were significantly different from what was obtained from cells cultured in basal medium supplemented with fasted pig sera (data not shown). There might be factors other than hormones contributing to adipogenic activity in serum. Since serum contains many components, some of which have been characterized, and others poorly characterized or not studied at all, the present results support the view of Loffler

and Hauner (1987) that there are yet unknown or undefined adipogenic factor (s) in serum. Thus, a 3 or 5-day fast may increase the levels or activities of some factor(s) in blood that normally promotes adipocyte differentiation and adipose conversion. The fact that fasted pig sera stimulated higher differentiation activity in primary culture remains intriguing and warrants further exploration.

The DNA data from primary cultures of rat S-V cells cultured in sera from fasted pigs indicates that there is a tendency for fasted pig sera not to support cell replication. Thyroid hormone levels are decreased in fasted pigs (Hodate et al., 1983; Buonomo and Baile, 1991) and thyroid hormone status influences adipocyte number in vivo (Picon and Levacher, 1979). Another regulatory hormone that can affect cell replication in this system is insulin-like growth factor-1 (IGF-1). 3T3-F442A Cells became sensitive to the mitogenic effect of IGF-1 after being exposed to growth hormone (Zezulak and Green, 1986). Fasting decreased circulating IGF-1 level despite an increase in plasma growth hormone (Buonomo and Baile, 1991). Ability of fasted pig serum to decrease muscle cell proliferation has been observed (White et al.,1988). Some serum-dependent changes in muscle cell proliferation in vitro have been attributed to decreased levels of circulating IGF-1 and increased levels and/or activities of factors that inhibit the proliferation of myogenic cells in serum of fasted pigs (White et al., 1988). An inhibitory factor in fasted pig serum has been partially purified (White et al., 1989). It is not known whether the same factors could play an inhibitory role in adipose S-V cell proliferation. Further studies are required using tritiated thymidine to see whether serum from fasted pigs inhibits S-V cell proliferation in culture.

Because fasting causes serum changes that affect adipose S-V cell differentiation and may induce changes that affect cell proliferation, investigation of changes in circulating factors that may affect adipocyte development in culture should increase our knowledge of the biology of adipocyte development in meat producing animals.

FIGURE 4.1. *Protein content of cultured porcine stromal-vascular cells obtained from adipose tissue of newborn pigs.* Cells were seeded on 6 well plates at a density of $3 \times 10^4$ cells/cm$^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% control pig serum (Fed), 2.5% 3 d fasted pig serum (3d F) or 2.5% 5 d fasted pig serum (5d F). Cellular protein content in cultured cells was determined on d 12. Values are means ± SE of four independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P<.05$.

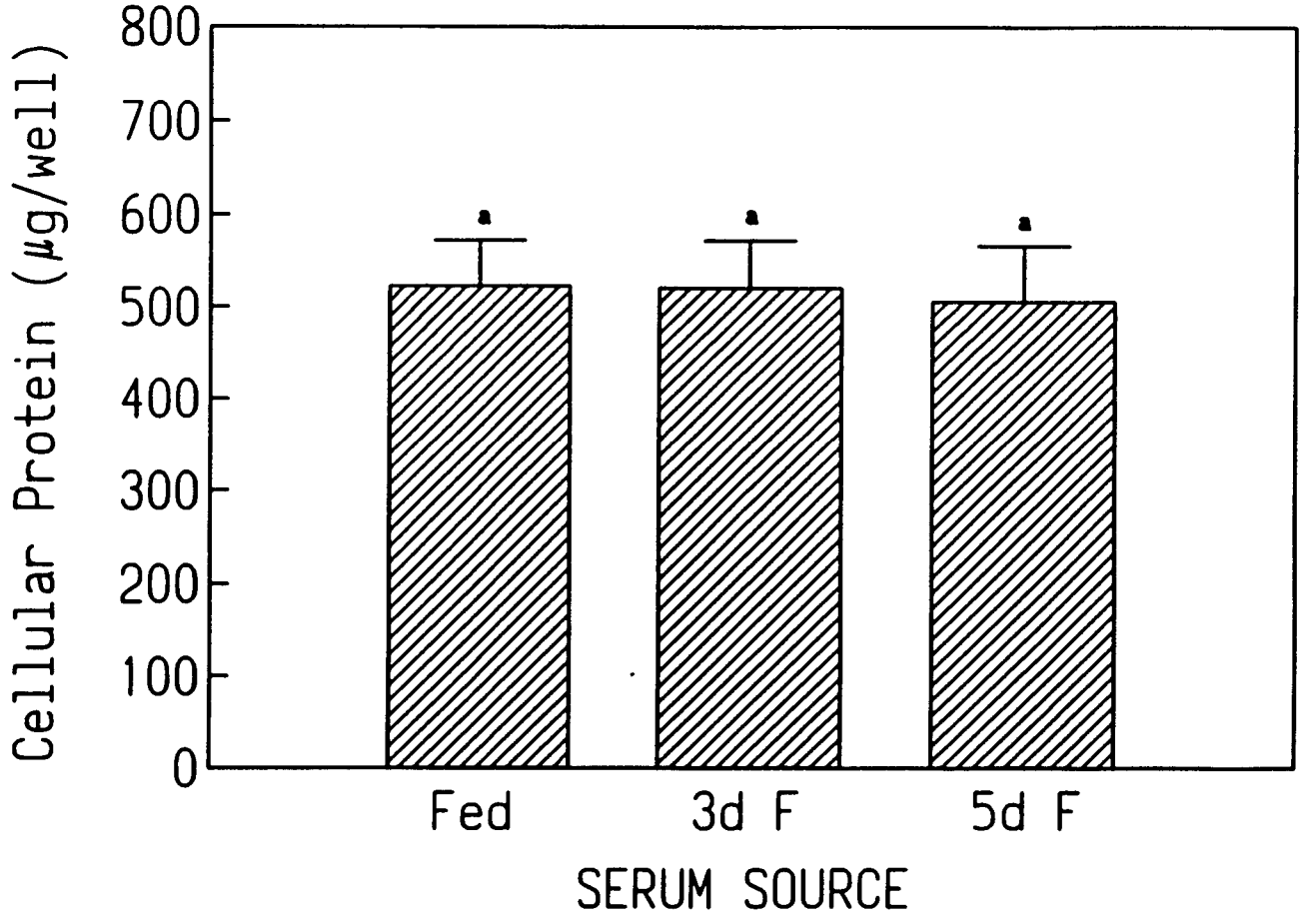
Cellular Protein (µg/well)
800
700
600
500
400
300
200
100
0
a
a
a
Fed
3d F
5d F
SERUM SOURCE

FIGURE 4.1

FIGURE 4.2. *Protein content of cultured rat stromal-vascular cells obtained from inguinal adipose tissue of male Sprague-Dawley rats.* Cells were seeded on 6 well plate at a density of 3 x $10^4$ cells/$cm^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% control pig serum (Fed), 2.5% 3 d fasted pig serum (3d F) or 2.5% 5 d fasted pig serum (5d F). Cellular protein content in cultured cells was determined on d 12. Values are means ± SE of four independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P < .05$.

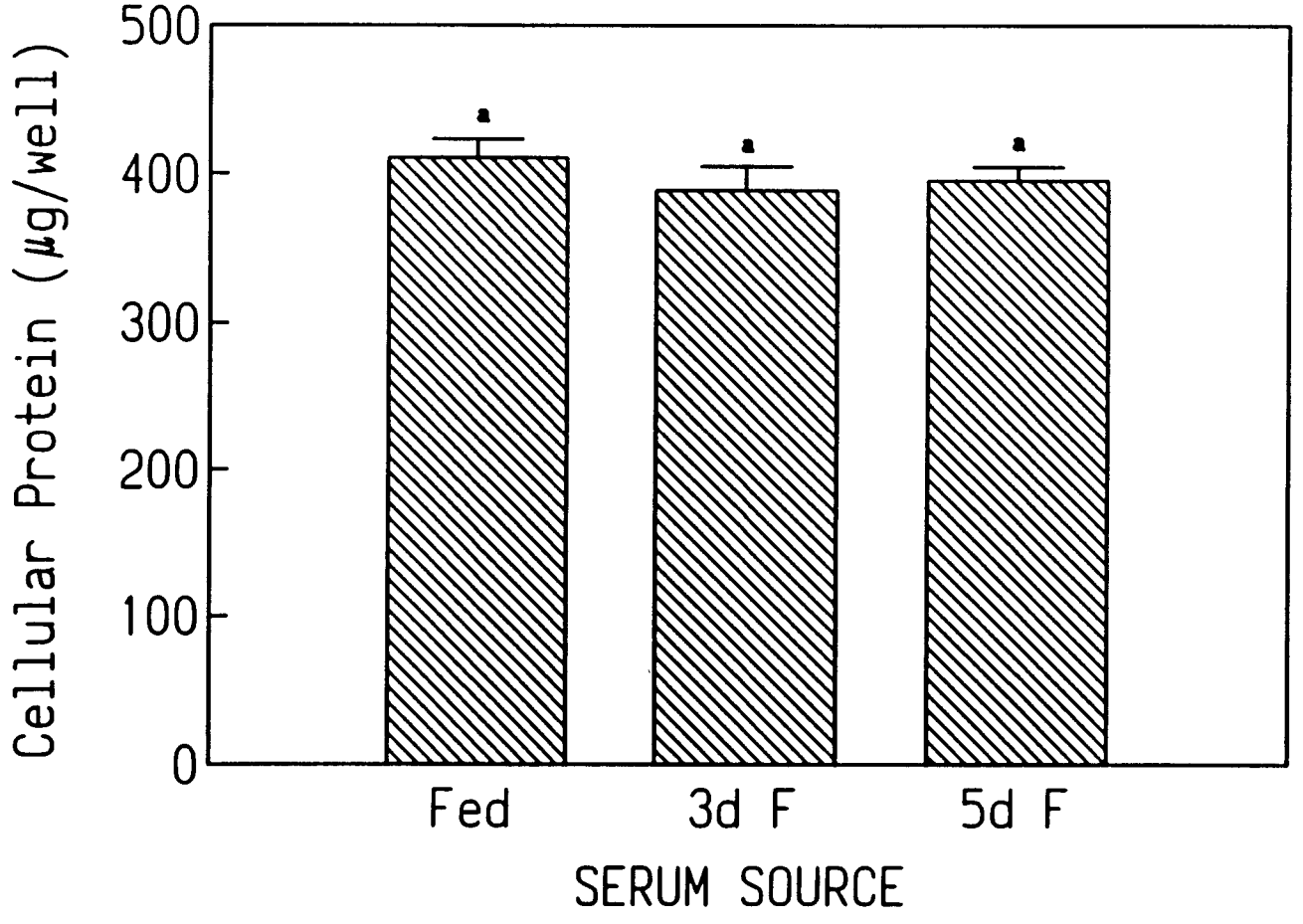
Cellular Protein (µg/well)
500
400
300
200
100
0
a
a
a
Fed
3d F
5d F
SERUM SOURCE

FIGURE 4.2

FIGURE 4.3. *DNA content of cultured porcine stromal-vascular cells obtained from adipose tissue of newborn pigs.* Cells were seeded on 6 well plates at a density of $3 \times 10^4$ cells/cm$^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% control pig serum (Fed), 2.5% 3 d fasted pig serum (3d F) or 2.5% 5 d fasted pig serum (5d F). DNA content in cultured cells was determined on d 12. Values are means ± SE of four independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P<.05$.

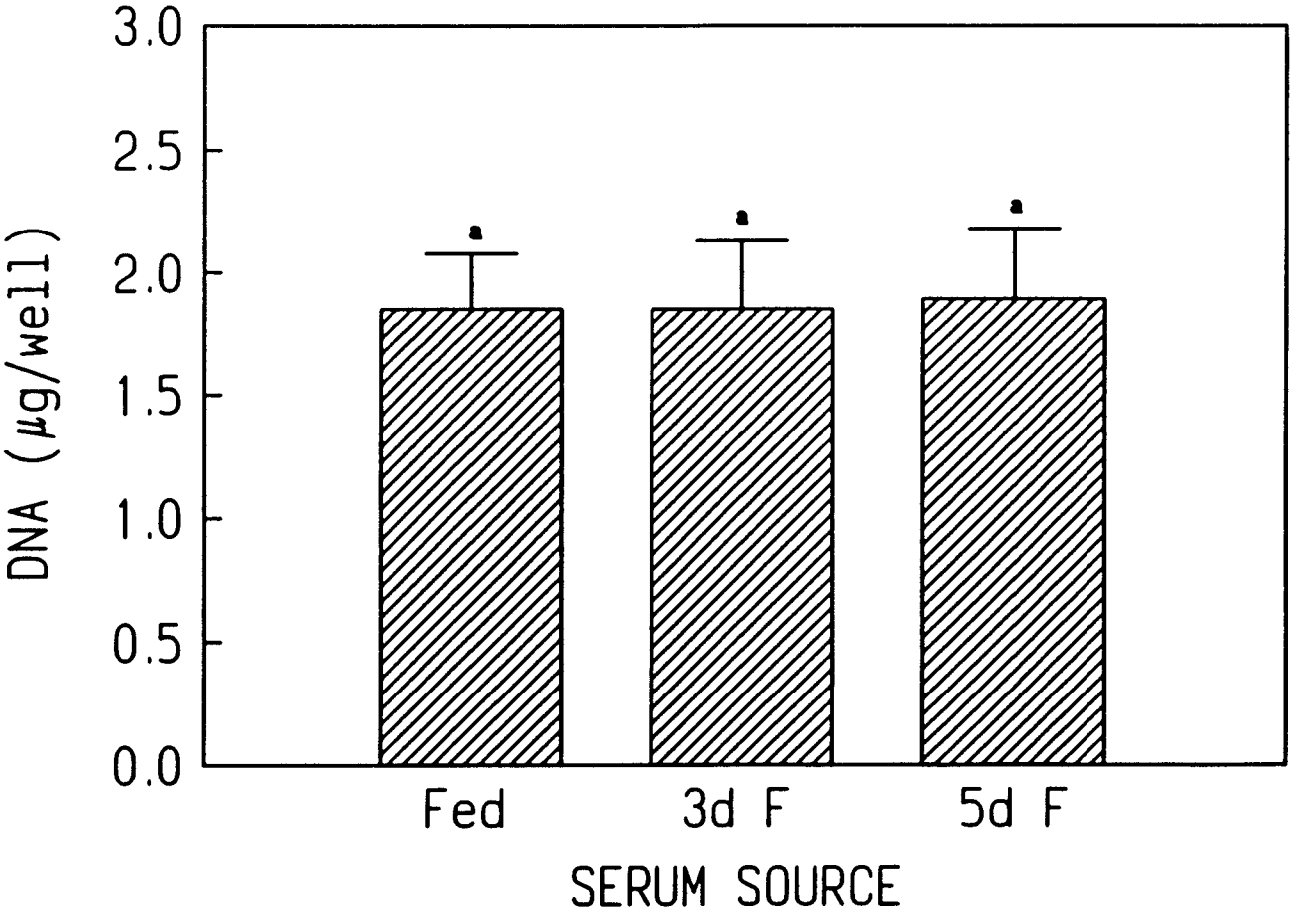


FIGURE 4.3

FIGURE 4.4. *DNA content of cultured rat stromal-vascular cells obtained from inguinal adipose tissue of male Sprague-Dawley rats.* Cells were seeded on 6 well plates at a density of 3 x $10^4$ cells/$cm^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% control pig serum (Fed), 2.5% 3 d fasted pig serum (3d F) or 2.5% 5 d fasted pig serum (5d F). DNA content in cultured cells was determined on d 12. Values are means ± SE of four independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P<.05$.

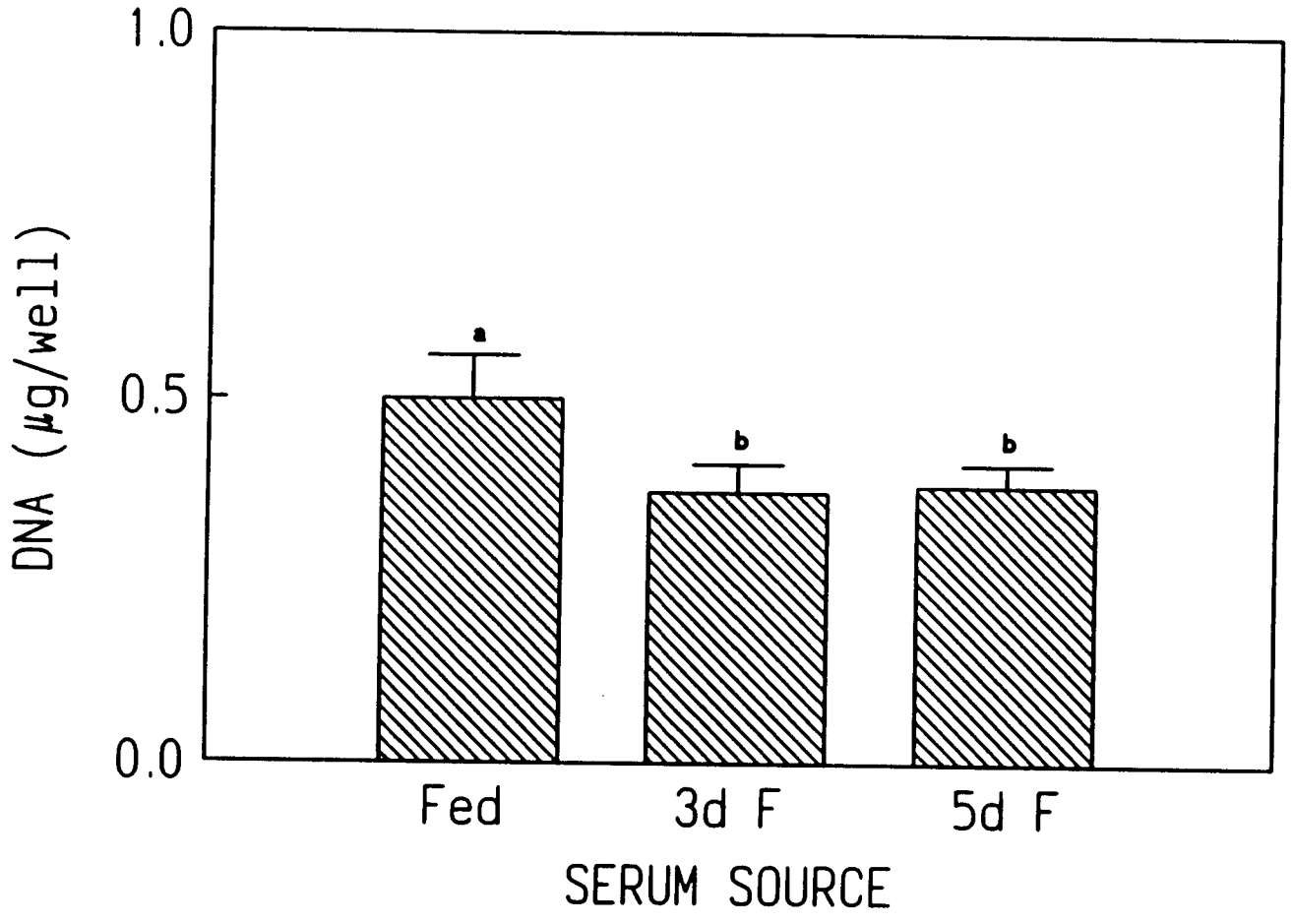


FIGURE 4.4

FIGURE 4.5. *Sn-glycerol-3-phosphate dehydrogenase activity in stromal-vascular cells obtained from adipose tissue of newborn pigs.* Cells were inoculated on 6 well plates at a density of $3 \times 10^4$ cells/cm$^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% control pig serum (Fed), 2.5% 3 d fasted pig serum (3d F) or 2.5% 5 d fasted pig serum (5d F). GPDH activity in cultured cells was determined on d 12. GPDH activity is expressed as percent of control (24.79 $\pm$ 4.51 nmoles/min/mg protein). Values are means $\pm$ SE of four independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P < .05$.

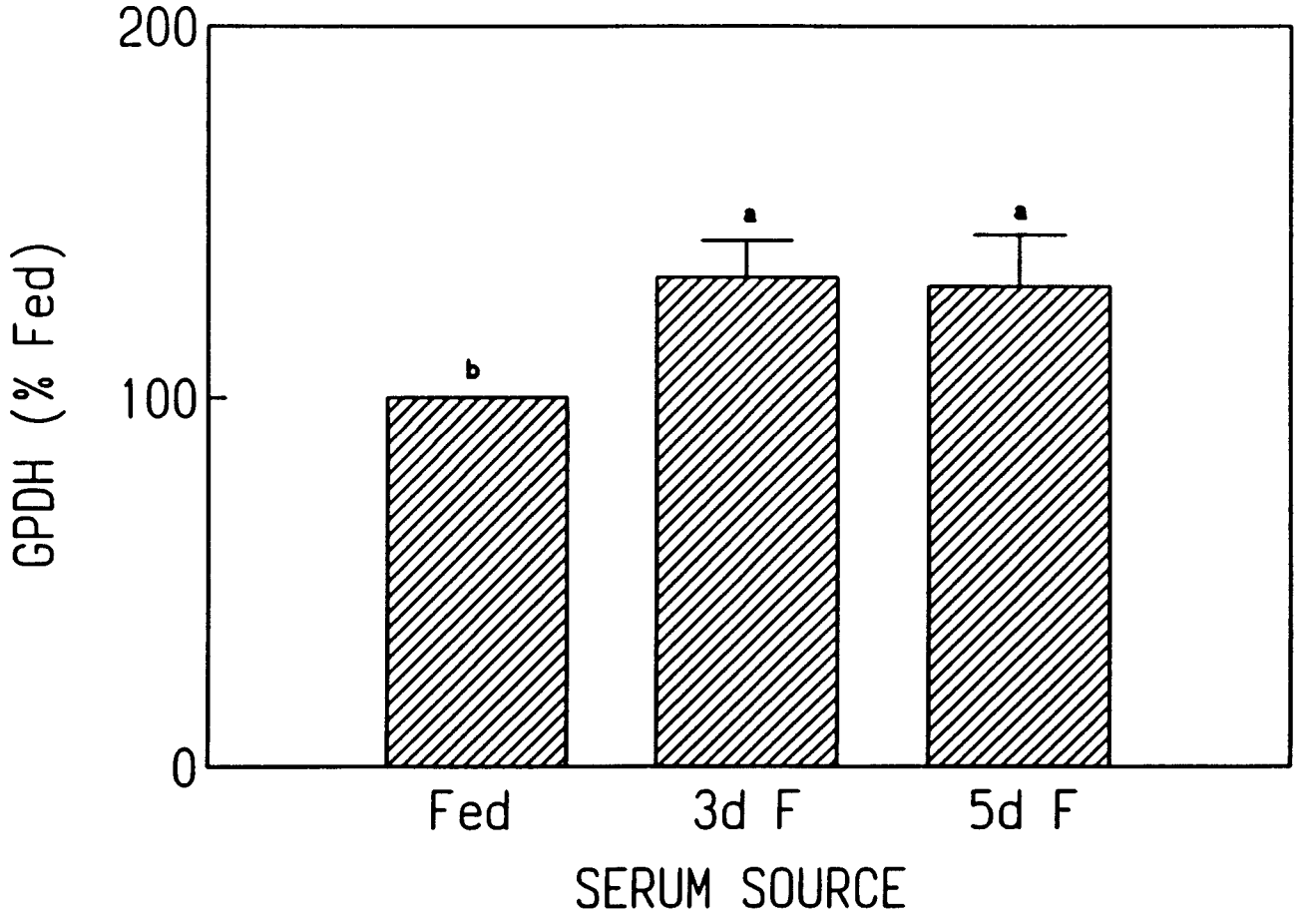


FIGURE 4.5

FIGURE 4.6. *Sn-glycerol-3-phospate dehydrogenase activity in stromal-vascular cells obtained from inguinal adipose tissue of male Sprague-Dawley rats.* Cells were inoculated on 6 well plates at a density of $3 \times 10^4$ cells/cm$^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% control pig serum (Fed), 2.5% 3 d fasted pig serum (3d F) or 2.5% 5 d fasted pig serum (5d F). GPDH activity in cultured cells was determined on d 12. GPDH activity is expressed as percent of control (117.74 ± 28.97 nmoles/min/mg protein). Values are means ± SE of four independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P < .05$.

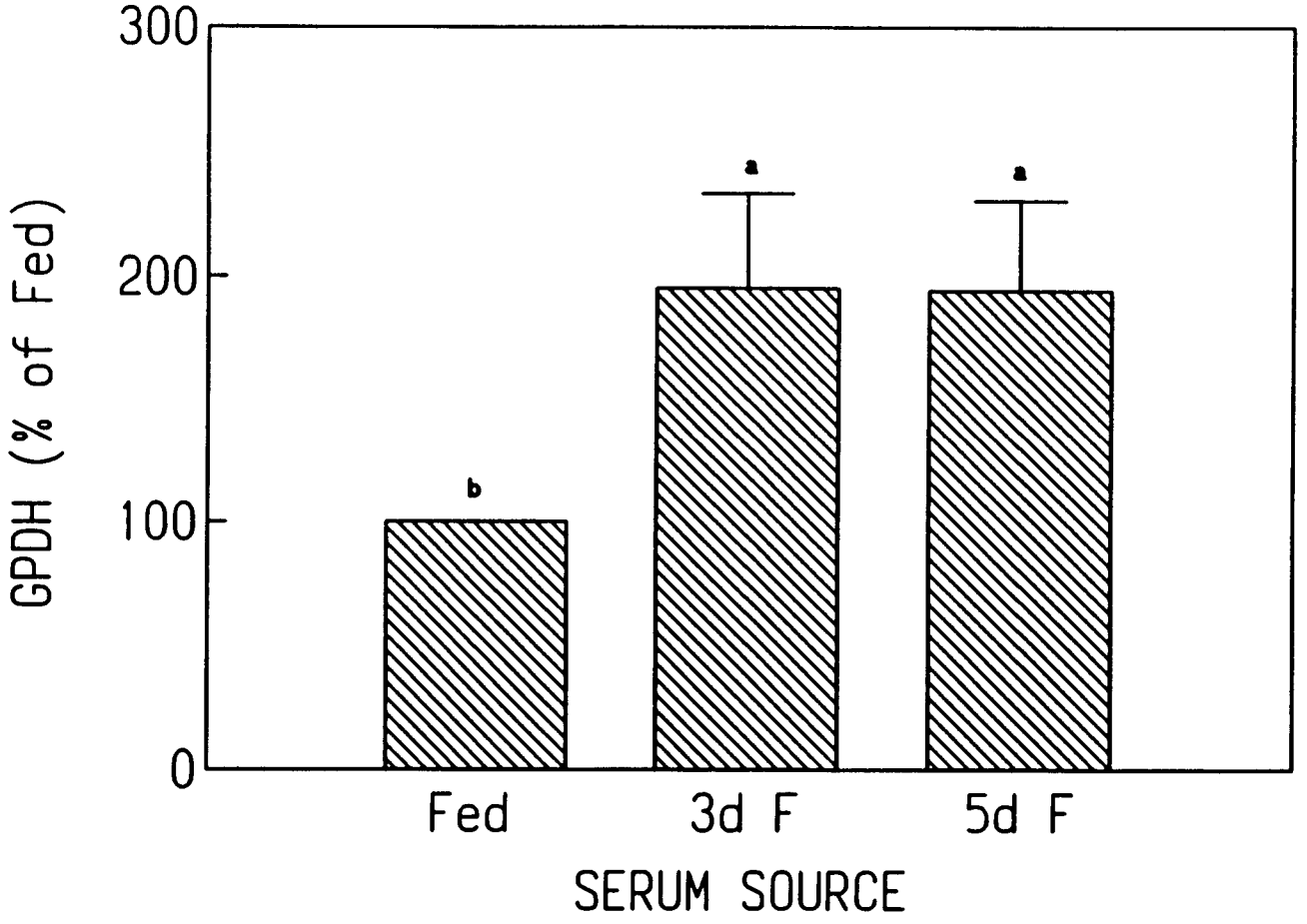


FIGURE 4.6

FIGURE 4.7. *Photomicrographs of pig adipose stromal-vascular cells cultured in sera from fed and fasted pigs.* Cells were inoculated at a density of 3 x $10^4$ cells/$cm^2$ and grown in DME/HAM's medium containing 10% FCS for 24 h after which cells were washed with DME/HAM's medium without FCS. Cells were subsequently grown in either DME/HAM's medium containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with (a) 2.5% control pig serum (Fed) and (b) 2.5% 3 d fasted pig serum (3d F). On day 12 of culture, cells were washed with phosphate buffered saline, fixed in 10% formalin, stained with oil red O and counterstained with hematoxylin. Cytoplasmic lipid droplets were stained red with oil red O while nuclei were stained blue with hematoxylin. Note the size of fat cell clusters formed in cultures grown in fasted pig serum. Magnification = 100x. Bar = 200 $\mu$.

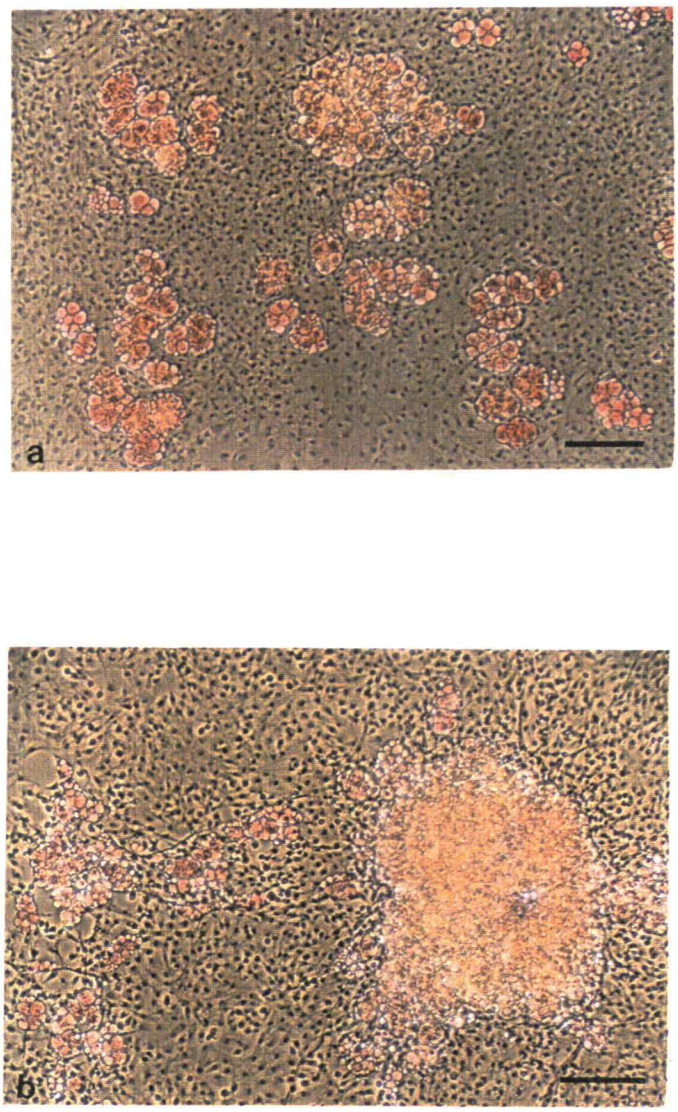


FIGURE 4.7

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# Chapter 5

## EFFECT OF SERA FROM LEAN AND OBESE PIGS ON THE DIFFERENTIATION OF PORCINE ADIPOSE STROMAL-VASCULAR CELLS IN CULTURE

## ABSTRACT

Primary cultures of stromal-vascular (S-V) cells from adipose tissue of newborn pigs were used to evaluate the characteristics of four sera obtained from male and female genetically lean or obese pigs weighing 50-60 Kg. Lean pig sera (LPS) stimulated greater levels of sn-glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) activity in cultured cells than obese sera (OPS). Male LPS tended to promote higher GPDH specific activity than female LPS or male and female OPS. When GPDH was expressed on per DNA basis, male LPS significantly ($P<.05$) increased GPDH per unit DNA compared to female LPS or male and female OPS. Cellular protein content per well was not significantly influenced by different sera. DNA content per well in cultures grown in female LPS was greater than in male LPS or female OPS. DNA per well in cultures grown in male OPS was higher than in cultures grown in female OPS or male LPS. These results demonstrate that lean pig serum differs from obese pig serum in its ability to promote differentiation of cultured porcine adipose S-V cells and further that this difference may be influenced by sex.

## INTRODUCTION

Genetically lean and obese pigs provide a model to study porcine growth, particularly deposition of carcass fat and accretion of muscle, and also provide a model, other than rodent models to study the physiology of obesity. Lean and obese strains of pigs were developed by Hetzer and Harvey (1967) by selecting for thick or thin backfat depth for about eighteen generations. Obesity can be detected in the pig model as early as 110 days of gestation (McNamara and Martin, 1982; Stone et al., 1985). Fat cell numbers are not different in lean and obese strains of pigs (Hausman et al., 1983). Morphologically, adipocytes from obese pigs are larger than adipocytes from lean pigs (Steele et al., 1974; Scott et al., 1981; Mersmann, 1986). Larger cell size in obese pigs might contribute to their higher body lipid deposition.

Blood metabolites in lean and obese pigs do not differ to a great extent. Plasma glucose, triglyceride and cholesterol concentrations are similar in neonatal and postnatal obese and lean pigs (Mersmann et al., 1982). Only slight difference exist in endocrine status of these pig strains. Plasma concentrations of growth hormone are lower, triiodothyronine are higher and cortisol is similar in late prenatal obese pigs as compared to lean pigs (Stone et al., 1985). Because of their unique endocrine and metabolite status the pig model of obesity is not compounded by major defects in carbohydrate and/or lipid metabolism as are most rodent models (Mersmann et al., 1982).

Sex of an animal affects adiposity through its endocrine profile (Seideman

et al., 1982). In mammals, the intact male has less carcass fat and more muscle than the intact female; the castrated male, on the other hand, has more fat and less muscle than the intact male and in some species, the female. There was increased lean tissue accretion when exogenous estrogenic compounds were administered to castrated male cattle and sheep (Schanbacher, 1984; Roche and Quirke, 1986).

Serum source profoundly influences proliferation and differentiation of adipose stromal-vascular cells in culture (Ramsay et al., 1987). 3T3-L1 cells have been used to investigate the effect of serum from genetically obese rodents on adipose conversion (Loffler et al., 1983). Similarly, rat adipose stromal-vascular cells have been used to investigate the effect of sera from genetically lean and obese pigs on rat preadipocyte differentiation in culture (Jewell and Hausman, 1988). It has been shown, however, that different growth responses are obtained when the test stromal-vascular cells and the test serum are from the same species than when they are obtained from different species (Jewell and Hausman, 1989). There are variations in the capacity of serum obtained from different animal species to support adipose conversion in culture (Kuri-Harcuch and Green, 1978) and there are variations in the ability of serum obtained from animals of the same species subjected to different treatments to promote differentiation of rat stromal-vascular cell differentiation in culture (Jewell et al., 1988). There are no reports on the effects of lean and obese pig sera on growth and development of porcine adipose stromal-vascular cells in culture. It is therefore pertinent to evaluate the ability of genetically lean and obese pig sera to promote the differentiation of stromal-vascular cells obtained from adipose tissue of pigs.

The objective of this study therefore, was to examine the effect of sera obtained from intact male and female obese and lean pigs on growth and development of porcine adipose stromal-vascular cells in culture.

## MATERIALS

Dulbecco's Modified Eagle's Medium (DME, D-5523), Nutrient mixture F-12 (HAM, N-6760, dihydroxy acetone phosphate (DHAP, D-7137), reduced nicotinamide adenine dinucleotide (NADH, N-8219), gentamicin sulfate (G-1264), hydrocortisone (H-0135), insulin (I-1882), triiodothyronine (T-5516), bovine transferrin (T-8027), and hematoxylin (HHS-2-16), were purchased from Sigma Chemical Co. (St Louis, MO). Bovine serum albumin (BSA, Bovuminar Reagent CRG-7) was purchased from Armour Pharmaceutical Co. (Tarrytown, NY); collagenase (type I) from Worthington Biochemical (Freehold, NJ); fetal calf serum (FCS) from Intergen Co., (Purchase, NY); Fungizone from Gibco BRL (Gaithersburg, MD); and Prepodyne from AMSCO, Medical Products Division (Erie, PA). All other reagents were of analytical grade.

## METHODS

### Animals

Newborn crossbred pigs obtained from a commercial producer were killed by $CO_2$ asphyxiation. Pigs were scrubbed thoroughly with Prepodyne and rinsed with 70% ethanol. Disinfected pigs were placed on sterile surgical tray in a laminar flow

hood. Incision was made in pig's skin from about .5 cm posterior to the base of the skull along the sagittal plane to the scapula and from the midline to about 3 cm laterally on both ends with a sterile scalpel. Adipose tissue sample in the exposed area was carefully removed from the underlying tissue with sterile forceps and scissors.

Stromal-Vascular Cell Isolation

Dissected adipose tissue samples were minced and incubated with digestion buffer, pH 7.4 at 37°C for 1h in a gyratory shaker. Digestion buffer contained 118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.3 mM $MgSO_4$, 10 mM $NaHCO_3$, 10 mM HEPES, 5 mM glucose, 40 mb/L gentamicin sulfate, 3% BSA, 2 mg/ml collagenase equilibrated with 95% $O_2$: 5% $CO_2$ and filter sterilized with 0.45 $\mu$m acrodisc filter (Gelman Sciences, Ann Arbor, MI). Ratio of tissue to buffer was 1 g to 3 ml. Digested tissue was filtered through a sterile single layer polyester chiffon and the infranatant was removed with a sterile syringe fitted with a long needle into a sterile 50 ml polypropylene centrifuge tube and centrifuged at 800 x g for 10 min. Stromal-vascular cell pellets were washed three times in DME/HAM's medium (1:1,v/v) containing 15 mM $NaHCO_3$, 15 mM HEPES buffer (pH 7.4), 40 mg/L gentamicin sulfate and 2 mg/L fungizone supplemented with 10% FCS (Plating medium).

Cell Culture

Aliquots of the stromal-vascular cells were removed, stained with Rappaport's stain and counted on a hemocytometer. Stromal-vascular cells were

seeded on Corning 6 well (35 mm) tissue culture plates in 3 ml of plating medium at a density of 3 x $10^4$ cells/$cm^2$. Cells were cultured at 37°C under a humidified atmosphere of 95% air: 5% $CO_2$; 24 hours later cells were washed extensively with plating medium without FCS to remove erythrocytes, other unattached cells and tissue debris. Cells were subsequently maintained in test media. Test media consisted of plating medium without FCS containing 20 $\mu$U/ml insulin, 1 ng/ml triiodothyronine, 25 ng/ml hydrocortisone, 10 $\mu$g transferrin (ITTC) supplemented with 2.5% treatment sera as indicated in figures and legends. Test media were changed every 3 days.

Sera Collection

Blood was collected by aortic puncture at time of slaughter from intact male and female lean and obese pigs weighing 50-60 kg. Blood was allowed to clot overnight at 4°C. After clotting, sera were separated by centrifugation and filter sterilized with 0.45 $\mu$m sterile acrodisc filter (Gelman Sciences, Ann Arbor, MI). Sera were pooled by genotype and sex before use in culture.

Enzyme Analysis

On day 12, activity of GDPH was measured in cell cultures by methods of Kozak and Jensen (1974) as modified by Wise and Green (1979).

DNA and Protein Content

DNA was assayed as described by LaBarca and Paigen (1980) using salmon

testes DNA as a standard. Protein content was determined by bicinchoninic acid (BCA) method using BSA as a standard (Pierce Chemical Co., Rockford, IL).

Statistical Analysis

The experimental design was a factorial arrangement of treatments in a randomized complete block (Steel and Torrie, 1980). Data were subjected to analysis of variance using the general linear models procedure and means differences were separated using contrast (SAS, 1987).

## RESULTS

The influence of serum on the protein content of cultured porcine stromal-vascular cells is shown in Figure 5.1. There was no influence of genotype, sex, and no genotype x sex interaction on the ability of serum to affect protein levels in cultured cells.

DNA contents of cultured porcine stromal-vascular cells as influenced by sera is shown in figure 5.2. DNA content of porcine stromal-vascular cells cultured in serum from lean male pigs (LM) was lower ($P<.05$) than DNA content of cells cultured in serum from lean female pigs (LF). The effect of serum from obese male (OM) and obese female pigs (OF) on cultured cell DNA levels was opposite that of their lean counterparts while there was no difference between DNA content in cultures fed LM and OF and in cultures fed LF and OM ($P>.05$).

GPDH activity in cultured porcine adipose stromal-vascular cells as influenced by sera is shown in Figure 5.3. There was no difference ($P>.05$) in GPDH activity

of cultures fed serum obtained from LM than in cultures fed serum obtained from LF. There was no difference in GPDH activity of stromal-vascular cells fed serum obtained from OM and OF. Serum obtained from LM stimulated higher GPDH activity in cultured cells than serum obtained from OM while serum obtained from LF stimulated higher GPDH activity than serum obtained from OF. Generally, serum obtained from lean pigs stimulated higher GPDH activity in cultured porcine stromal-vascular cells than serum obtained from obese pigs. When GPDH activity was expressed on per DNA basis, cells cultured in serum obtained from LM had higher ($P < .05$) GPDH per DNA than cells cultured in serum obtained from LF. There was no difference between GPDH activity per DNA basis in cells cultured in the different obese sera ($P > .05$).

## DISCUSSION

In vivo studies using radiolabelled thymidine incorporation into adipose tissue have indicated that newly filled adipocytes are from a population of cells containing newly synthesized nuclear DNA (Hollenberg and Vost, 1968; Greenwood and Hirsch, 1974; Gaben-Cogneville and Swierczewski, 1979). Growth and development of stromal-vascular cells in culture provides us with a tool to examine the effects of growth factors, hormones or sera on differentiation and proliferation of preadipocytes (Hauner and Loffler, 1987). In the present study primary culture system was used to evaluate differences between sera obtained from different strains and different sexes of pigs on the differentiation of stromal-vascular cells obtained from pigs.

Changes in serum borne components or factors control preadipocyte growth and development (Ramsay et al., 1987). Obese and lean pigs have similar levels of plasma glucose, triglyceride and cholesterol at birth and throughout the postnatal period. Thus, growing obese pigs are not hyperglycemic, hypertriglyceridemic or hypercholesterolemic as are other animal obesity models (Mersmann et al., 1982). This means that obese pigs are metabolically normal when compared to their lean counterparts with regards to lipid and/or carbohydrate metabolism. Serum from genetically obese pigs stimulated higher differentiation activities than serum from lean pigs in primary culture of rat adipose stromal-vascular cells (Jewell and Hausman, 1988). Similarly, serum from genetically obese rodents stimulated greater adipocyte differentiation in 3T3-L1 cells (Loffler et al., 1983). The results of the present study are in contrast with the reports of Jewell and Hausman, (1988) and Loffler et al. (1983). Jewell and Hausman (1988) utilized stromal-vascular cells obtained from rat adipose tissue while Loffler et al. (1983) used adipocyte-like cell line developed from Swiss mouse embryo. It has been shown that subsequent growth responses of cultured cells was affected by species of the test stromal-vascular cells as well as the serum (Jewell and Hausman, 1989). The discrepancies in the results reported by Jewell and Hausman (1989) and Loffler et al. (1983) and the present results might be due to differences in cells to which treatments were applied. Since lipogenic substrates in the sera of lean and obese pigs are similar (Mersmann et al., 1982), the increase in GPDH activity in cultures treated with serum from lean pigs was not a result of changes in lipogenic substrates in the medium.

Divergence in body composition between lean and obese pigs can be seen as early as 110 d of gestation; body fat content is slightly higher in obese than lean pigs (Stone et al., 1985). Adipose tissue lipogenic rates are higher in growing obese than lean pigs (Steele et al., 1974; Steele and Frobish, 1976; Scott et al., 1981). Metabolic differences between lean and obese strains of pigs may be reflected in plasma concentrations of hormones.

Plasma levels of growth hormone are lower, triiodothyronine are higher and cortisol are the same in obese compared to lean pigs (Stone et al., 1985). This endocrine profile in obese pigs would support adipogenesis, and the divergence in body composition between lean and obese could reflect endocrine-genetic influence. Strong adipogenic response of cultured stromal vascular cells used in this study suggests that there may be an inherent defect in the adipocyte of obese pigs in vivo since stromal-vascular cells used in this study were obtained from contemporary pigs and the confounding effects of disease, changing hormonal levels, activity and genotype in intact animals are mostly eliminated. Lean and obese pig sera produced divergent responses in porcine adipose stromal-vascular cells in cultures. Specific factors responsible for adipogenic activity in pig models of obesity have not been identified. These results suggest that there are unknown adipogenic factor(s) in sera of lean and obese pigs, the levels and/or activities of which might be higher in lean than obese pig sera. The levels or activities of these factors in lean pigs might be influenced by sex since there was tendency for LM to stimulate higher differentiation activity in cultured cells than LF. Since lean pig sera stimulated greater differentiation than their obese counterparts it is likely that increase in

adipose tissue mass in obese pigs is due more to intrinsic than to extrinsic influences. Very few metabolic and hormonal differences exist between genetically obese and lean pigs (Mersmann et al., 1982; Stone et al; 1985), suggesting that adipocytes in lean and obese pigs are different. This is supported by report of Killefer and Hu (1990) who found an adipocyte specific plasma membrane protein to be present in genetically lean pig adipocytes and absent in genetically obese pigs.

Serum borne factors in lean and obese pigs do not differ in their ability to support cellular growth since there was no difference in cellular protein levels in cultured cells. However, the DNA data indicate that there might be variation in the levels or activities of mitogenic components in lean and obese pig sera and the levels or activities might be influenced by sex. Since the increase in DNA content in cell cultures did not correspond to increase in differentiation activity, expressed as GPDH specific activity or as GPDH per unit DNA, the response of cultured cells may reflect a greater number of differentiation activity per cell as opposed to a greater number of responsive preadipocytes. At present it can be concluded that most of the increase in cell number or DNA content is attributable to replication of other cell types rather than preadipocytes. More definitive studies are needed to identify adipogenic serum fractions and other yet unidentified growth factors in sera of obese and lean pigs and to determine the possible association of adipocyte-specific proteins with obesity. These will delineate between intrinsic and extrinsic influences on extremes in porcine growth, particularly regarding lipid deposition and protein accretion in the body.

FIGURE 5.1. *Soluble protein content in porcine adipose stromal-vascular cells cultured in lean or obese pig serum.* Cells were seeded on 6 well plates at a density of $3 \times 10^4$ cells/cm$^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h in order to facilitate cell attachment to the wells. After 24 h cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% of serum obtained from lean male (LM), lean obese (LO), lean female (LF), or obese female (OF) pigs. Protein content of cultured cells was determined on day 12. Values are means $\pm$ SE of five independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P<.05$.

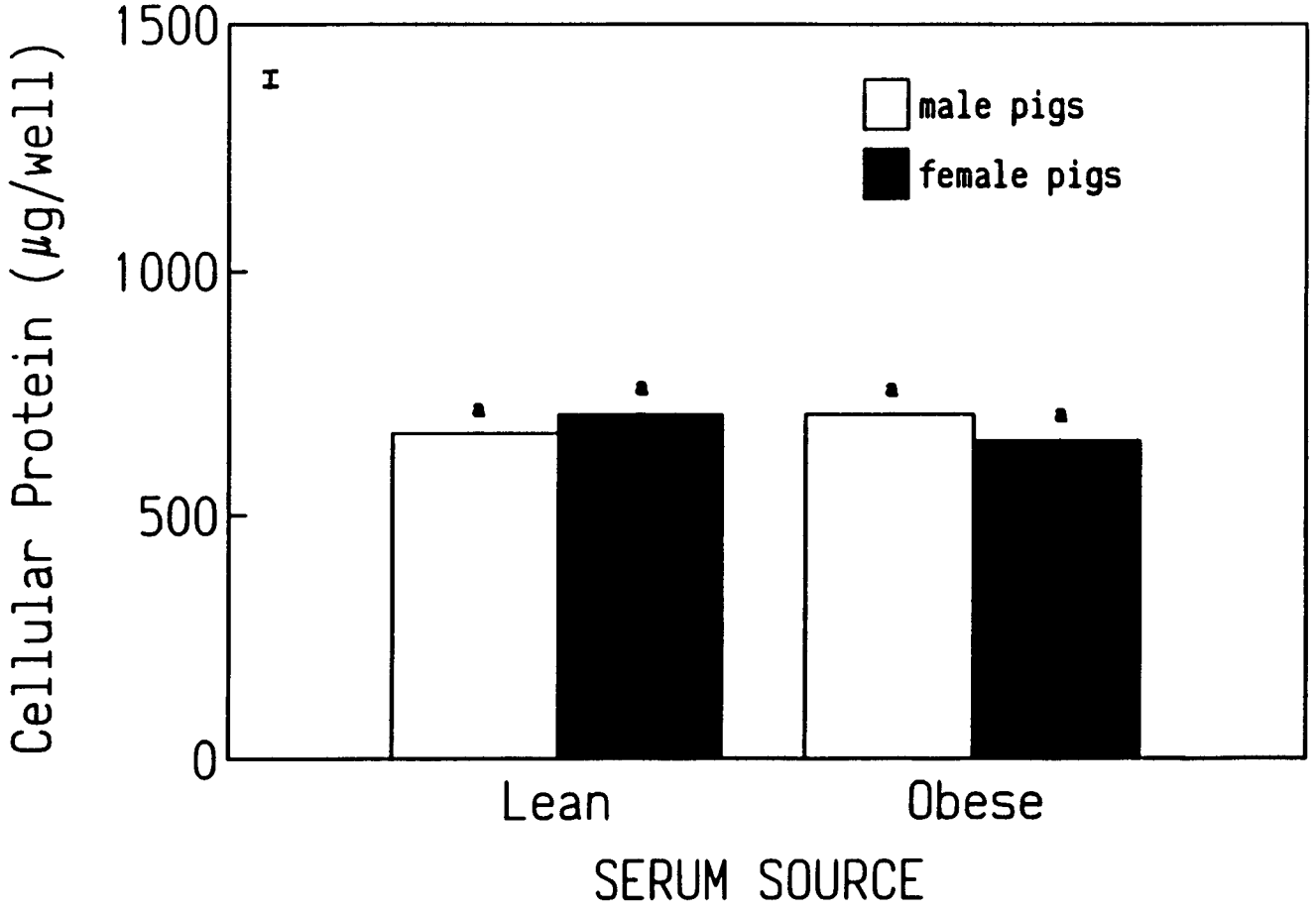
1500
1000
500
0
Cellular Protein (µg/well)
male pigs
female pigs
a
a
a
a
Lean
Obese
SERUM SOURCE

FIGURE 5.1

FIGURE 5.2. *DNA content of porcine adipose stromal-vascular cells cultured in lean or obese pig serum.* Cells were seeded on 6 well plates at a density of $3 \times 10^4$ cells/$cm^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h in order to facilitate cell attachment to the wells. After 24 h cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% of serum obtained from lean male (LM), lean obese (LO), lean female (LF), or obese female (OF) pigs. DNA content of cultured cells was determined on day 12. Values are means ± SE of five independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P < .05$.

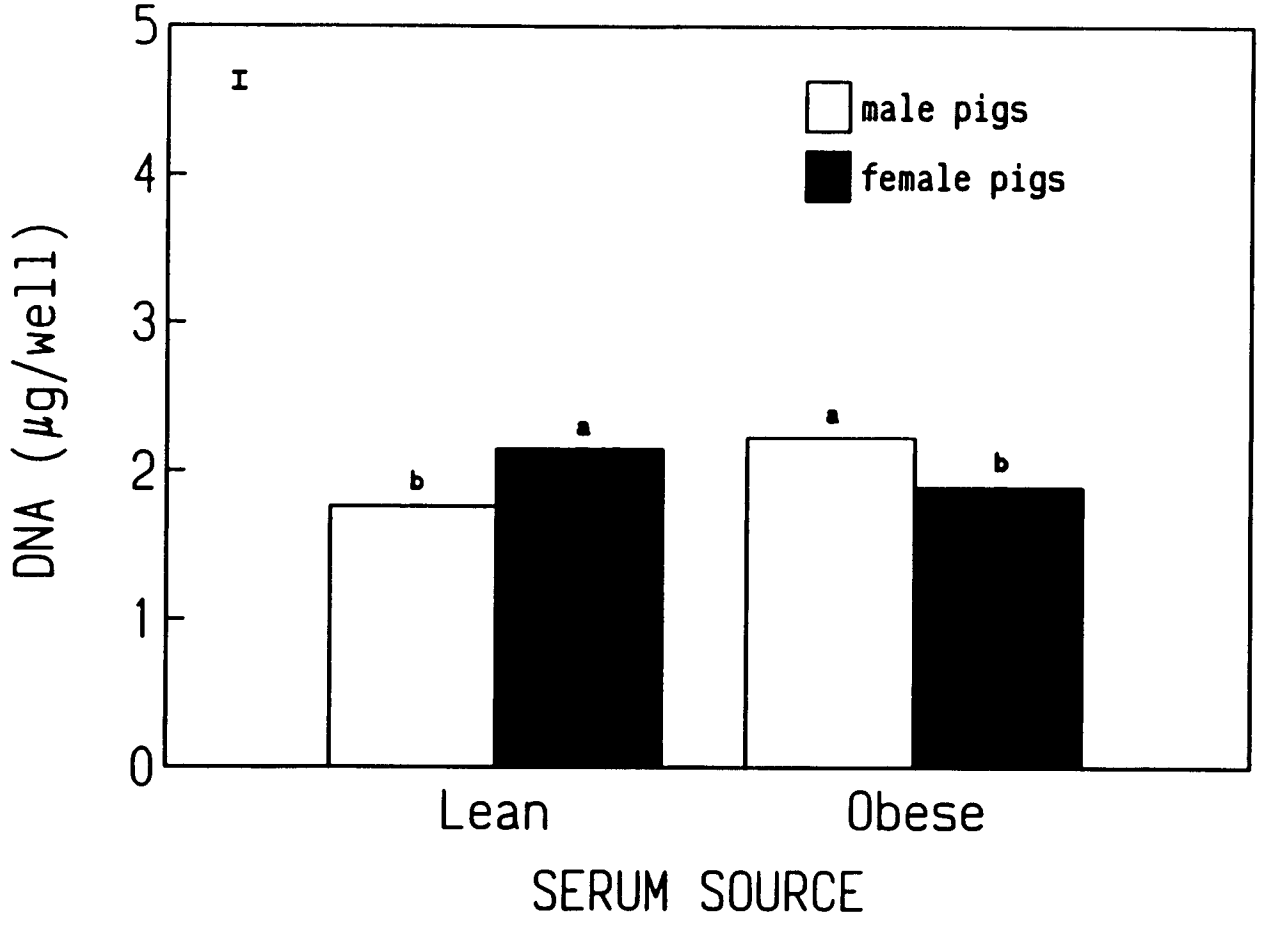
I
male pigs
female pigs
DNA (µg/well)
5
4
3
2
1
0
b
a
a
b
Lean
Obese
SERUM SOURCE

FIGURE 5.2

FIGURE 5.3. *Sn-glycerol-3-phosphate dehydrogenase activity in stromal-vascular cells cultured in lean or obese pig serum.* Cells were seeded on 6 well plates at a density of 3 x $10^4$ cells/$cm^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h in order to facilitate cell attachment to the wells. After 24 h cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% of serum obtained from lean male (LM), lean obese (LO), lean female (LF), or obese female (OF) pigs. GPDH activity in cultured cells was determined on day 12 and expressed as (A) specific activity and (B) activity per unit DNA. Values are means ± SE of five independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P < .05$.

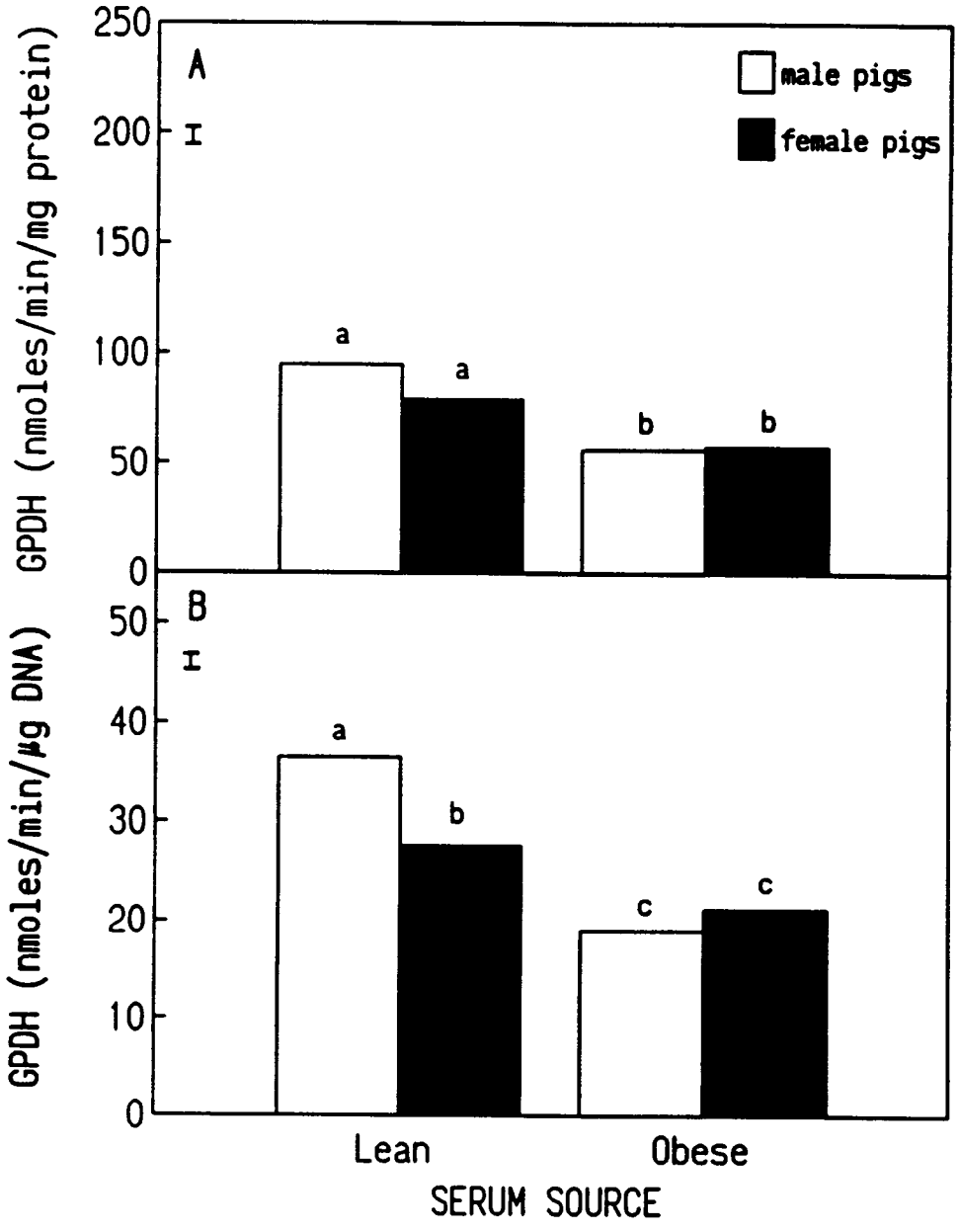
GPDH (nmoles/min/mg protein)
GPDH (nmoles/min/µg DNA)
A
B
male pigs
female pigs
a
a
b
b
a
b
c
c
250
200
150
100
50
0
50
40
30
20
10
0
Lean
Obese
SERUM SOURCE

FIGURE 5.3

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# Chapter 6

# EFFECT OF SERUM SOURCE ON DIFFERENTIATION OF CULTURED ADIPOSE STROMAL-VASCULAR CELLS

## ABSTRACT

Primary cultures of stromal-vascular (S-V) cells from adipose tissue of rats and pigs were used to investigate the differences in preadipocyte development in response to sera from different pigs and rats. Cells were seeded at 3 x $10^4$ cells/$cm^2$ and cultured for 12 days in medium supplemented with 2.5% serum from different strains of rats or serum from pigs of differing ages. Differentiated pig S-V cells developed as individual cells when cultured in serum free medium and developed as a discrete cluster of cells when cultured in serum supplemented medium. Differentiated rat S-V cells developed as individual cells in either serum free or serum containing medium. Rat cells cultured in Sprague-Dawley or Fischer rat serum had greater GPDH per unit DNA than pig cells cultured in the same medium. There was no difference in GPDH activity in pig cells cultured in serum from pigs of differing ages, but serum from 3 month old pig stimulated higher GPDH activity in rat S-V cells than serum from newborn or 6 month old pig. Cell replication was greater in cultures of pig cells grown in pig or rat serum than rat cells grown in the same medium as indicated by DNA numbers. GPDH activity was higher in rat epididymal compared rat inguinal derived S-V cells. These results suggest that pig and rat S-V cells are morphologically different and they respond differently to the same stimuli. Anatomic site influences the differentiation of rat S-V cells in culture.

## INTRODUCTION

The primary cultures of stromal-vascular (S-V) cells isolated from adipose tissue of various species including man have shown the existence of cells capable of proliferating and acquiring adipocyte phenotype and biochemical characteristics (Bjorntorp et al., 1978; Hauner et al., 1989; Ramsay et al., 1989). Similarly, established adipocyte-like cell lines are able to undergo differentiation in culture into mature adipocytes (Ailhaud, 1982; Spiegelman et al., 1988). Using cell culture systems, the ability of several sera to influence adipocyte development has been investigated. Kuri-Harcuch and Green (1978) found that there are variations in the ability of sera from different species to promote differentiation of mouse clonal cell line, 3T3-F442A cells. Fetal bovine serum stimulated more adipose conversion than postnatal bovine serum and other sera tested due to the presence of more adipogenic factor in fetal bovine serum (Kuri-Harcuch and Green, 1978). Primary cultures of S-V cells have been used to evaluate the in vitro effect of sera and plasma of different species on differentiation and adipose conversion of rat preadipocytes (Bjorntorp et al., 1985). Evaluation of most sera for adipogenic activity has been carried out using rat S-V cells. Jewell and Hausman (1988) found that both rat and pig S-V cells responded to growth and differentiation factors in rat and pig serum but the levels of response were different. Rat S-V cell cultures had higher glycerol-3-phosphate dehydrogenase activity, an adipocyte differentiation marker, than pig cells cultured in the same medium. There are also morphological differences between rat and pig S-V cells in culture (Jewell and Hausman, 1989).

Tume et al. (1985) found species specific differences in polypeptide composition of adipocyte plasma membranes. Differences in membrane proteins may cause differences in growth and function of cells. The relevance of using cells obtained from one species to evaluate adipogenic activity of serum from another species may be questionable.

The carcass of newborn piglets contains about 2% fat (Seerley et al., 1974, 1978) and much of the fat content of newborn piglets is structural and is not available as energy (Mersmann, 1974). Robison (1976) studied growth patterns in swine and found that fat deposition in carcass is almost linearly associated with age. Younger pigs (about 25 Kg) are accreting mostly muscle mass while older pigs (about 50 Kg) are at stage of growth when there is concomitant increase in carcass muscle and adipose deposition but with accelerating rate of adipose accretion. Cell environment can influence cell growth, it is not known whether the differences in adipose cellularity in neonatal and growing pigs are influenced by serum borne factors. Since there are differences in the ability of sera from different species to influence development of adipocyte in culture and there are differences in the morphological development of rat and pig S-V cells in culture, the objectives of this study were to investigate the growth response of rat and pig S-V cells cultured in serum from either rat or pig and to examine whether the divergence in adipose cellularity in pig at different stages of growth is influenced by serum factors.

## MATERIALS

Dulbecco's Modified Eagle's Medium (DME, D-5523), nutrient mixture F-12 (HAM, N-6760), dihydroxy acetone phosphate (DHAP, D-7137), reduced nicotinamide adenine dinucleotide (NADH, N-8219), gentamicin sulfate (G-1264), hydrocortisone (H-0135), insulin (I-1882), triiodothyronine (T-5516), bovine transferrin (T-8027), hematoxylin (HHS-2-16), were purchased from Sigma Chemical Co.(St. Louis, MO). Bovine serum albumin (BSA, Bovuminar Reagent CRG-7) was purchased from Armour Pharmaceutical Co. (Tarrytown,NY); collagenase, (type I) from Worthington Biochemical (Freehold, N.J.); fetal calf serum (FCS) from Intergen Co., (Purchase, N Y); Prepodyne from AMSCO, Medical Products Division (Erie, PA) and Fungizone from Gibco BRL (Gaithersburg, MD). All other reagents were of analytical grade.

## METHODS

### Animals

Crossbred pigs less than one day old were obtained from a commercial producer and male Sprague-Dawley rats weighing 160-180 g were purchased from Simonsen Laboratories, Inc. (Gilroy, CA). Both newborn pigs and Sprague-Dawley rats served as cell donors.

Sera Collection

Blood was collected from 6 newborn, 8 three month (50 Kg) and 8 six month (100 Kg) old pigs by venipuncture of the anterior vena cava. Blood was collected from Sprague-Dawley rat by heart puncture. Blood was allowed to clot overnight at 4 °C. After clotting, the sera were separated by centrifugation and filter sterilized using 0.45 $\mu$m sterile Acrodisc, low protein binding filter (Gelman Sciences, Ann Arbor, MI). Sera were pooled by species and age for use in culture. Three month old male Fischer 344 rat serum was obtained from Hilltop Lab Animals, Inc. (Scottdale, PA).

Stromal-vascular cell Isolation

Animals were killed by $CO_2$ asphyxiation, scrubbed with Prepodyne and rinsed with 70% ethanol solution. Disinfected pig or rat was placed on sterile surgical tray in a laminar flow hood. Incision was made in pig's skin from about .5 cm posterior to the base of the skull along the sagittal plane to the scapula and from the midline to about 3 cm laterally on both ends with a sterile scalpel. Adipose tissue sample in the exposed area was carefully removed from the underlying tissue with sterile forceps and scissors. For rats, incision was made at the ventral skin transversely at the median line just over the diaphragm with sterile scissors. The skin was cut longitudinally, grasping the skin on both sides of the cut with two pairs of sterile forceps the skin was pulled in the opposite directions to expose the ventral surface of the body. Inguinal fat pads on both thighs were removed from

the underlying tissue, and the epididymal fat was dissected out with sterile forceps and scissors. Dissected tissue samples were put in separate petri dishes containing 118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.3 mM $MgSO_4$, 10 mM HEPES, 5 mM glucose and 40 mg/L gentamicin sulfate, equilibrated with 95% $O_2$:5% $CO_2$ and sterilized by filtering through 0.45 $\mu$m Nalgene disposable syringe filter (Nalge Co., Rochester, NY). Adipose tissue samples (1 g) were minced with a pair of sterile scissors and digested for 1 h at 37 °C in a gyratory water bath in a 25 ml polypropylene flask containing 3 ml KRB buffer with 3% BSA and 2 mg/ml collagenase that has been filter sterilized. Digested tissue was filtered through a sterile single layer polyester chiffon and cell suspension was centrifuged at 800 x g for 10 min. Stromal-vascular (S-V) pellets were washed three times in DME/HAM's medium (1:1, v/v) containing 15 mM $NaHCO_3$, 15 mM HEPES buffer (pH 7.4), 40 mg/L gentamicin sulfate and 2 mg/l Fungizone supplemented with 10% FCS (Plating medium)

Cell Culture

Aliquots of the S-V cells were removed, stained with Rappaport's stain and counted on a hemocytometer. S-V cells were seeded in plating medium on Corning 6 well (35 mm) tissue culture plates at a density of 3 X $10^4$ cells/$cm^2$. Cells were cultured at 37 °C under a humidified atmosphere of 95% air:5% $CO_2$; 24 hours later cells were washed 2 x 5 min and 1 x 1 h with plating medium without FCS to remove unattached materials, mainly erythrocytes and tissue debris. Cells were subsequently maintained in test media. Test media consisted of plating medium

without FCS, plus 20 μU/ml insulin, 1 ng/ml triiodothyronine, 10 μg/ml transferrin and 25 ng/ml hydrocortisone (ITTC) supplemented with 2.5% Sprague-Dawley or Fischer rat serum or serum from pigs of differing ages. Test media were changed every 3 days until day 12 when cultures were terminated and protein, DNA and Sn-glycerol-3-phosphate dehydrogenase were determined.

Enzyme Analysis

Sn-glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8 was measured by spectrophotometric methods of (Kozak and Jensen, 1974) as modified by Wise and Green (1979).

DNA and Protein Content

DNA was assayed as described by LaBarca and Paigen (1980) using salmon testes DNA as a standard. Protein content was determined by bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as a standard (Pierce Chemical Co., Rockford, IL).

Histochemistry

Representative wells from each treatment were fixed in 10% formalin, stained with oil red O for lipid and counterstained with Harris hematoxylin (Boon and Drijver, 1986) after 12 d of exposure to test media.

Statistical Analysis

This experiment used factorial arrangement of treatments in a complete randomized design (Steel and Torrie, 1980). Data were subjected to analysis of variance according to General Linear Models Procedure and contrast between means were determined (SAS, 1987).

## RESULTS

Porcine S-V cells grown in DME/HAM's medium supplemented with 2.5 or 10% rat or pig serum did not maintain a smooth monolayer. On day three in culture cells became retractile and created several blank spots on culture plate (Fig. 6.1). After a day or two, cells eventually clumped together and lifted off the culture plate surface. In contrast cells maintained in DME/HAM's medium plus 10 $\mu$U/ml insulin, 1 ng/ml triiodothyronine, 10 $\mu$g/ml transferrin and 25 ng/ml hydrocortisone (SF) did not exhibit the characteristic described for cell cultured in DME/HAM's medium with serum. Cells in SF remained attached to culture support throughout the length of the culture period (Fig. 6.1) as did cells cultured in SF supplemented with 2.5 or 10 % rat or pig serum. Cell were well attached and maintained a smooth monolayer; as a result subsequent cultures were performed in SF supplemented with test sera.

When porcine S-V cells were grown in SF alone differentiated cells developed as individual cells, while they developed as discrete cluster in serum supplemented medium (Fig. 6.1). Small lipid droplets can be seen in differentiated porcine S-V cells on day 3 in culture medium. As differentiated cells became bigger, lipid droplets in individual cell's cytoplasm increased and became larger by day 12. Rat

S-V cells on the other hand developed as individual adipocytes when they differentiated in SF medium or SF supplemented with serum. Like porcine S-V cells small lipid droplets can be seen in the cytoplasm of differentiated cells on day 3 in culture. By day 12 differentiated rat S-V cell cytoplasm, unlike the porcine cell, was filled with a single fat globule instead of numerous lipid droplets (Fig. 6.2).

The effect of Sprague-Dawley (SR) or Fischer (FR) rat serum on cellular protein content in pig or rat S-V cells is shown in Figure 6.3. Serum from either SR or FR rat did not alter cellular protein content of cultured rat or pig cells. Pig cells cultured in SF medium supplemented with either SR or FR rat serum had higher ($P<.05$) DNA content than rat cells cultured in the same medium (Fig. 6.3).

The influence of serum from pigs of differing ages on protein levels in S-V cells from rat or pig is shown in Figure 6.4. Pig cells cultured in newborn pig (NB) or 3 month old pig (MS) serum had greater ($P<.05$) levels of cellular protein than rat cells cultured in the same medium. There was no difference in protein content of rat and pigs cells cultured in 6 month old pig (PS). DNA content of cultured cells in serum from pigs of differing ages is shown in Figure 6.4. Pig cells had higher ($P<.05$) DNA contents than rat cells.

Cellular protein was independent of serum source but was higher ($P<.05$) in S-V cells from inguinal adipose tissue cultured in SR serum than S-V cells from epididymal adipose tissue cultured in the same medium. DNA levels in inguinal and epididymal derived S-V cells cultured in the same medium did not differ, but either SR or FR serum promoted greater ($P<.05$) DNA synthesis in cultured cells than PS (Fig. 6.5).

Figure 6.6 shows the GPDH activity in rat or pig S-V cells cultured in SF supplemented with 2.5% SR or FR. GPDH specific activity in rat or pig S-V cells cultured in SR or FR rat serum was not different ($P < .05$). When GPDH activity was expressed on per unit DNA, pig cells cultured in either SR or FR rat serum had higher ($P < .05$) GPDH per unit DNA than rat cells cultured in the same medium.

When rat or pig cells were cultured in serum from pigs of differing ages, GPDH specific activity was higher ($P < .05$) in rat S-V cells cultured in NB or MS serum than pig S-V cells cultured in the same medium, but pig cells cultured in 6 PS had higher ($P < .05$) GPDH specific activity than rat cells cultured in the same medium (Fig. 6.7). When GPDH activity was expressed on per unit DNA basis, rat cells had higher ($P < .05$) GPDH per unit DNA than pig cells cultured in the same medium (Fig. 6.7).

GPDH specific activity in rat S-V cells was affected by adipose tissue anatomic site. S-V cells from epididymal adipose tissue of rat had higher ($P < .05$) GPDH activity than S-V cells from inguinal adipose tissue cultured in the same medium (Fig. 6.8).

## DISCUSSION

This study used S-V cells from pig and rat adipose tissue to evaluate differences in growth response to sera from their own species or to sera from other species. Newborn pig S-V cells cultured in 10% fetal pig serum did not maintain monolayer (Hentges and Hausman, 1989). In the present study newborn pig S-V

cells cultured in 2.5% of newborn pig or Sprague-Dawley rat serum produced effect similar to what was reported by Hentges and Hausman (1989). In addition, pig S-V cells in the present study failed to adhere to culture support after changing the medium from DME/HAM's medium supplemented with 10% FCS to DME/HAM's medium supplemented with test sera. This phenomenon did not allow the culture period to last for more than 3 or 4 days. Three or 4 days of culture would not permit the expression of GPDH enzyme marker for adipocyte differentiation (Pairault and Green, 1979) and its measurement in cultured cells. GPDH activity rises after lag phase and maximum activity is reached about 20 days (Schmidt et al., 1990). Since pig S-V cells cultured in serum free hormone supplemented (SF) medium did not exhibit this characteristic (retraction), serum free medium was used as basal medium to evaluate the characteristics of the test sera. It is not known whether this growth pattern is a characteristic of pig S-V cells or whether there is a serum factor causing this event. The present observations however eliminate intrinsic cell factor from consideration since pig cells did not exhibit this characteristic when cultured in SF alone or SF supplemented with sera.

Pig and rat S-V cells in culture are morphologically different. The present observations are in agreement with the reports of Jewell and Hausman (1989). Throughout the culture period differentiated pig S-V cells remained multilocular whereas differentiated rat S-V cells changed from multilocular to unilocular. Differentiated rat S-V cells contain numerous small lipid droplets in the first stage of adipose conversion and proceed to become cells with one, two, or three prominent fat globules in the terminal stage of maturation (Gregoire et al., 1990).

The present results on the morphology of adipose conversion in rat S-V cells is similar to what was described by Gregoire et al. (1990). These results suggest that there are morphological differences between differentiated rat and pig S-V cells under the present culture conditions. May be the nutrient requirements are different; the culture condition is optimal for rat cells but not pig cells.

Kuri-Harcuch and Green (1978) reported that there are variations in the ability of sera from different species to stimulate differentiation and adipose conversion of 3T3-F442A. Fetal bovine serum stimulated greater differentiation and adipose conversion than other sera tested. Rat serum stimulated higher differentiation in primary culture of rat adipose S-V cells than other sera tested, and the ability of rat serum to promote greater adipose conversion was attributed to a protein factor in rat serum (Li et al., 1989). This rat serum growth factor may be species specific. Adipogenic factors influencing differentiation and adipose conversion of cell lines and primary cultures of S-V cells are different (Li et al., 1989). Therefore, when studying the regulation of preadipocyte differentiation, caution should be taken when extrapolating from cell line data, as various factors acting as adipogenic factors in cell lines may be different in primary cultures. Since adipogenic factors in rat serum may be species specific (Li et al., 1989) and since rat and pig S-V cells in culture are morphologically different, the present study used both rat and pig S-V cells to evaluate serum from different rats and pigs. Pig and rat cells have similar GPDH specific activity but rat cells have greater GPDH per unit DNA than pig cells, indicating that rat cells have more clones that can differentiate in culture than pig cells. Pig cells replicated faster than rat cells when cultured in

sera from either Fischer or Sprague-Dawley strains of rats. Since the increase in cell number in cultured pig cells as indicated by DNA content per dish did not significantly contribute to number of differentiated cells, this suggests that the proliferated cells in pig S-V cells were non-preadipocytes.

Rat and pig S-V cells responded differently to growth stimuli in sera from pigs of differing ages. Serum obtained from 3 month old pigs showed highest differentiation activity in rat cells. Pig cells responded similarly to the sera from all three pig ages. This indicates that growth response of cells may be different if grown in serum from the same species than it would be in serum from different species. DNA content in pig cells cultured in sera from all three pig ages are higher that rat cells cultured in the same medium. Since rat cells had higher differentiation activity than pig cells, the increase in cell number in pig cell cultures as indicated by DNA content is attributable to multiplication of other cell types rather than preadipocytes. At present it can be concluded that cell intrinsic activity rather than serum factors may be responsible for divergence in cellularity of neonatal and mature pig adipose tissue as determined by differentiation of pig S-V cells in culture.

The results of the present study are in agreement with the report of Ramsay et al. (1987) who found that postnatal pig serum stimulated higher differentiation and lipid filling in cultures of rat S-V cells than fetal pig serum. However, since cultures of pig S-V cells did not produce the same effect as rat S-V cells cultured in similar medium, caution should be taken when extrapolating from in vitro data from rat to in vivo situations in pigs.

Several laboratories have demonstrated the influence of anatomic site of

differentiation and replication of rat S-V cells in culture. Perirenal derived S-V cells replicated and differentiated more extensively than epididymal cells under similar culture conditions (Djian et al., 1983; Kirkland et al., 1990). Gregoire et al. (1990) found that S-V cells from inguinal adipose tissue contained higher GPDH activity than S-V cells from epididymal adipose tissue. The present results also indicate that anatomic site influences replication and differentiation of rat S-V cells in culture. However, the results are in contrast with report of Gregoire et al. (1990) who found that GPDH activity in cultured S-V cells from inguinal adipose tissue was higher than GPDH activity in S-V cells from epididymal. The discrepancy between the present results and those of Gregoire et al. (1990) might be due to culture conditions employed. The present study cultured 3 X $10^4$ cells/$cm^2$ in DME/HAM's medium containing insulin, triiodothyronine, and hydrocortisone supplemented with 2.5% Sprague-Dawley or Fischer rat or pig serum as compared to 0.5 X $10^4$ cells/$cm^2$ in DME containing insulin and 10% fetal bovine serum used by Gregoire et al. (1990). Variations in ability of different sera and variations in the response of different cells to stimuli in serum may be responsible for the differences in results. The results of the present study suggest that S-V cells from different species interact differently and produce different effect with serum from their own species than with serum from other species. Further studies are required to identify the factors causing difference in response cell from one species to growth factors in serum of other species.

FIGURE 6.1. *Photomicrographs of pig adipose stromal-vascular cells cultured in serum free or serum containing medium.* Cells were inoculated at a density of 3 x $10^4$ cells/$cm^2$ and grown in DME/HAM's medium containing 10% FCS for 24 h. after which cells were washed with DME/HAM's medium without FCS. Cells were subsequently grown in DME/HAM's medium supplemented with 2.5% pig serum (a), 2.5% Sprague-Dawley rat serum (b), 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine (SF, c) and SF supplemented with 2.5% pig serum (d). On day 12 of culture, cells were washed with phosphate buffered saline, fixed in 10% formalin, stained with oil red O and counterstained with hematoxylin. Cytoplasmic lipid droplets were stained red with oil red O while nuclei were stained blue with hematoxylin. Note the cell retraction in culture grown in either 2.5% pig or rat serum. Magnification = 100x. Bar = 200 $\mu$.

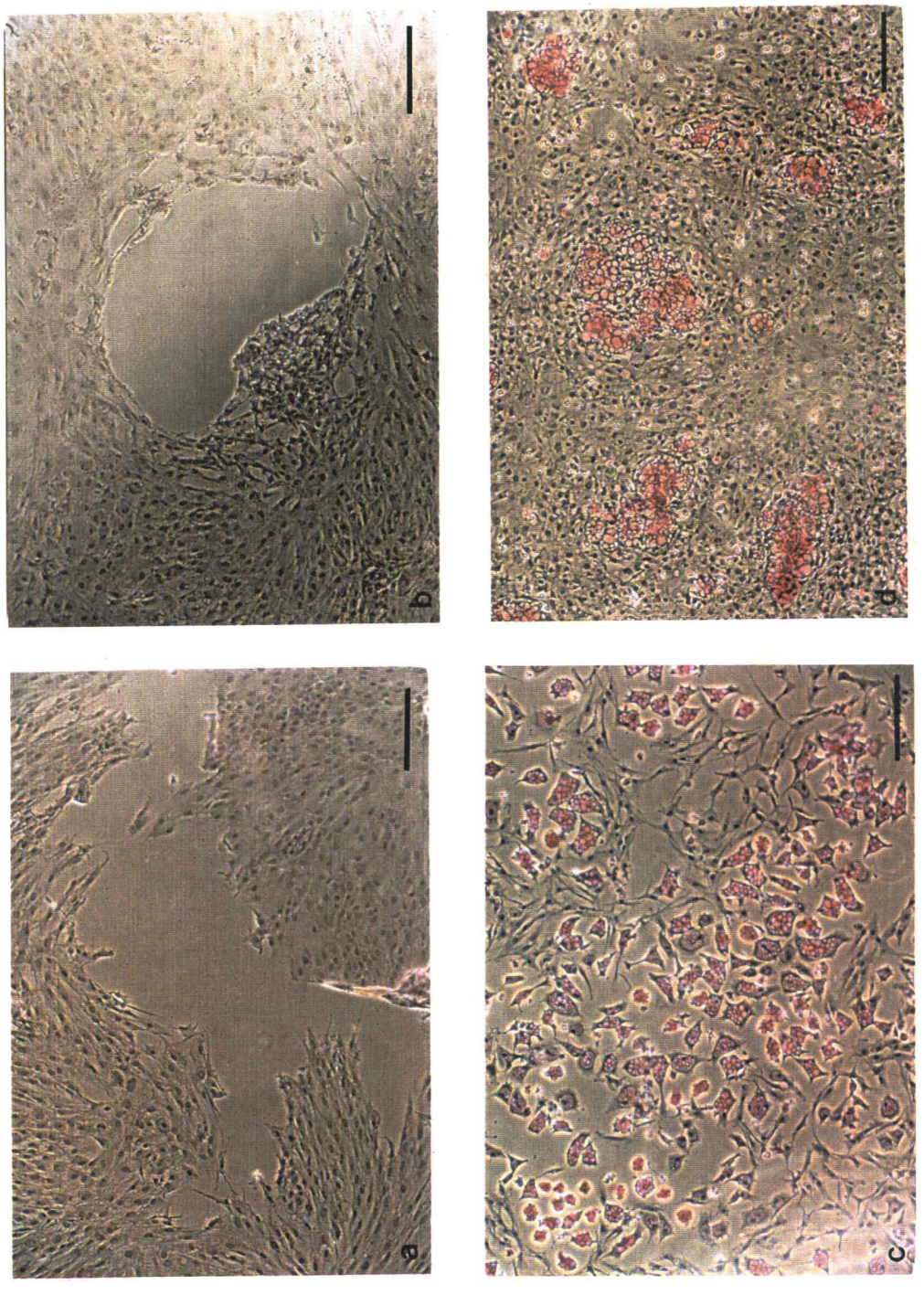



FIGURE 6.1

FIGURE 6.2. *Photomicrographs showing morphology of pig or rat adipose stromal-vascular cells cultured in serum free or serum containing medium.* Cells were inoculated at a density of 3 x $10^4$ cells/$cm^2$ and grown in DME/HAM's medium containing 10% FCS for 24 h after which cells were washed with DME/HAM's medium without FCS. Cells were subsequently grown in DME/HAM's medium supplemented with 2.5% pig or Sprague-Dawley rat serum or 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine (SF). Pig cells grown in SF for 12 days (a), pig cells grown in SF supplemented with 2.5% pig serum for 12 days (b), rat cells grown in SF for 3 days (c), rat cells grown in SF for 12 days (d), rat cells grown in SF supplemented with 2.5% Sprague-Dawley rat serum for 3 days 3 (e) and rat cells grown in SF supplemented with 2.5% Sprague-Dawley rat serum for 12 days (f). On day 3 or 12 of culture, cells were washed with phosphate buffered saline, fixed in 10% formalin, stained with oil red O and counterstained with hematoxylin. Cytoplasmic lipid droplets were stained red with oil red O while nuclei were stained blue with hematoxylin. Differentiated pig cells appeared as individual cells in SF and as a cluster of cells in SF plus pig serum on day 12. Note that differentiated rat cells appeared as individual cells on day 3 or 12 in SF or SF supplemented with serum. Magnification = 100x. Bar = 200 $\mu$.

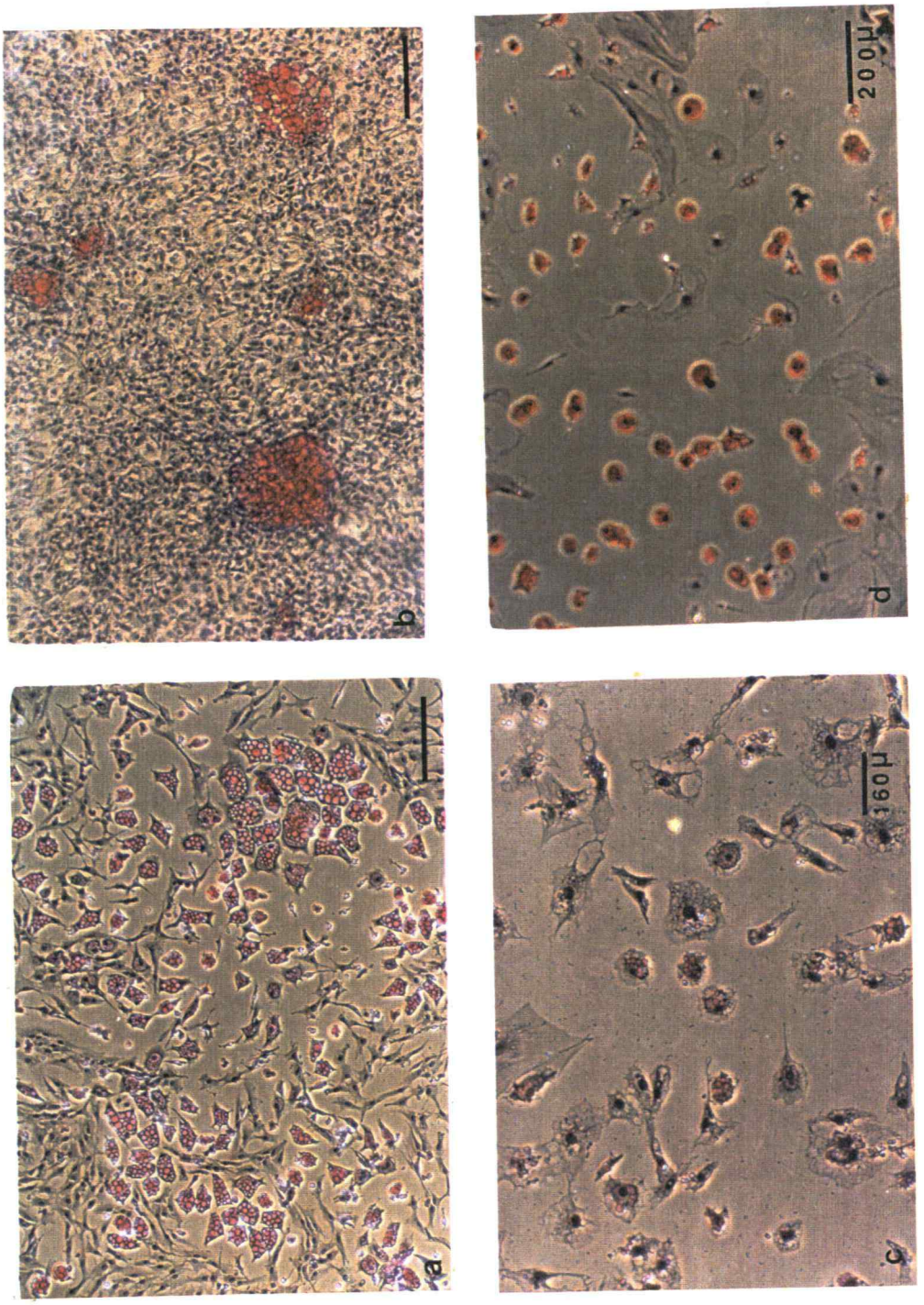



FIGURE 6.2

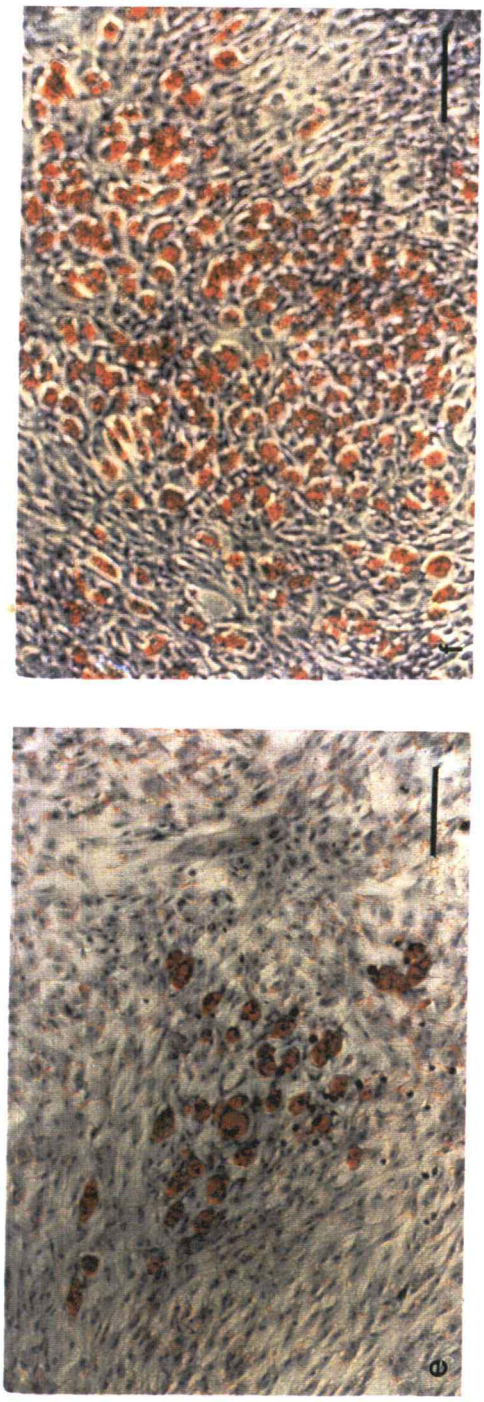
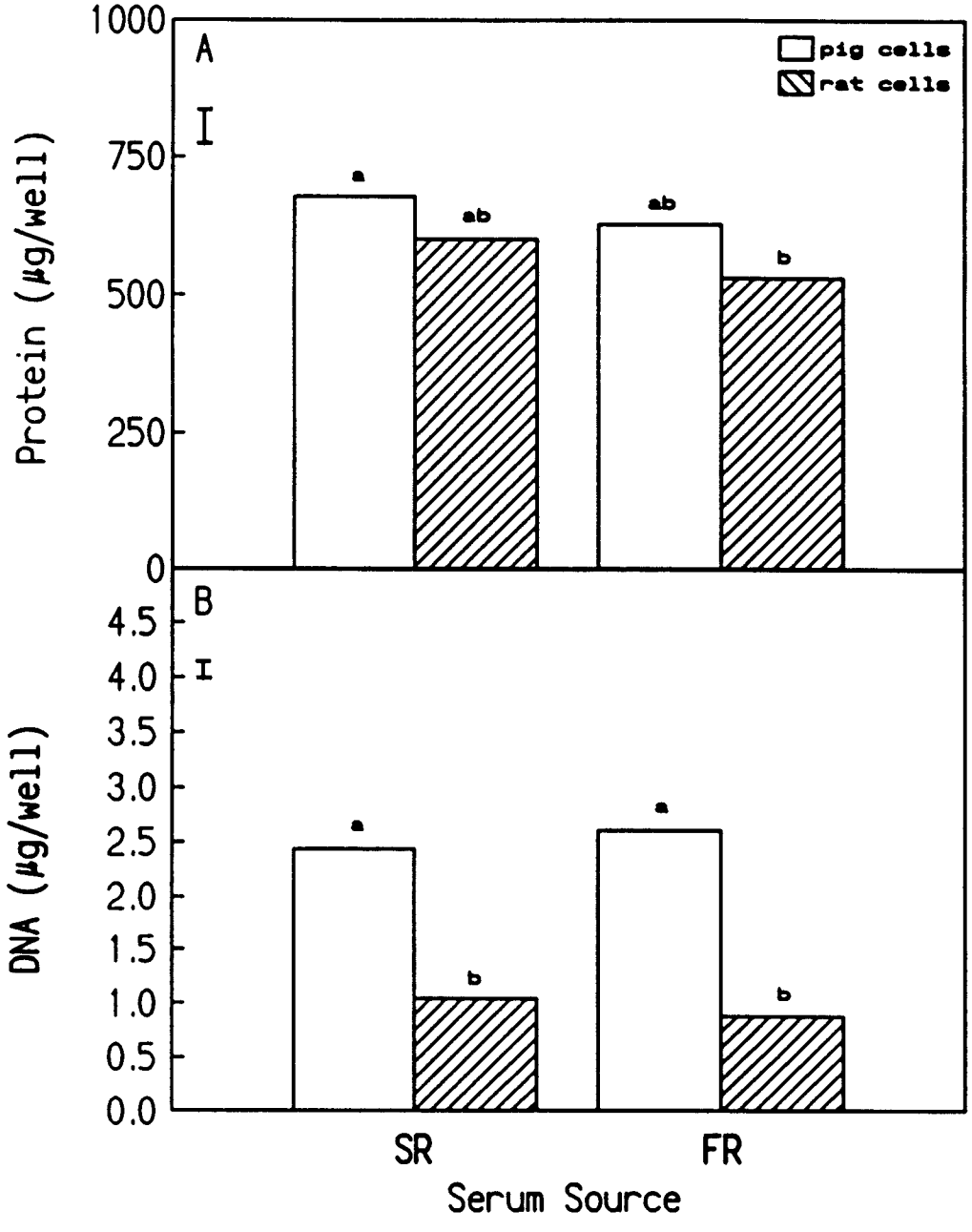


Figure 6.2 Continued.

FIGURE 6.3. *Cellular protein and DNA contents of cultured stromal-vascular cells obtained from rat or pig adipose tissue.* Cells were seeded on 6 well plates at a density of $3 \times 10^4$ cells/cm$^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% Sprague-Dawley rat (SR) or Fischer rat (FR) serum. Cellular protein and DNA contents in cultured cells were determined on d 12. Values are means of four independent experiments performed on triplicate wells. Bar represents pooled standard error. Means with the same letter are not significantly different at $P < .05$.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pig cells
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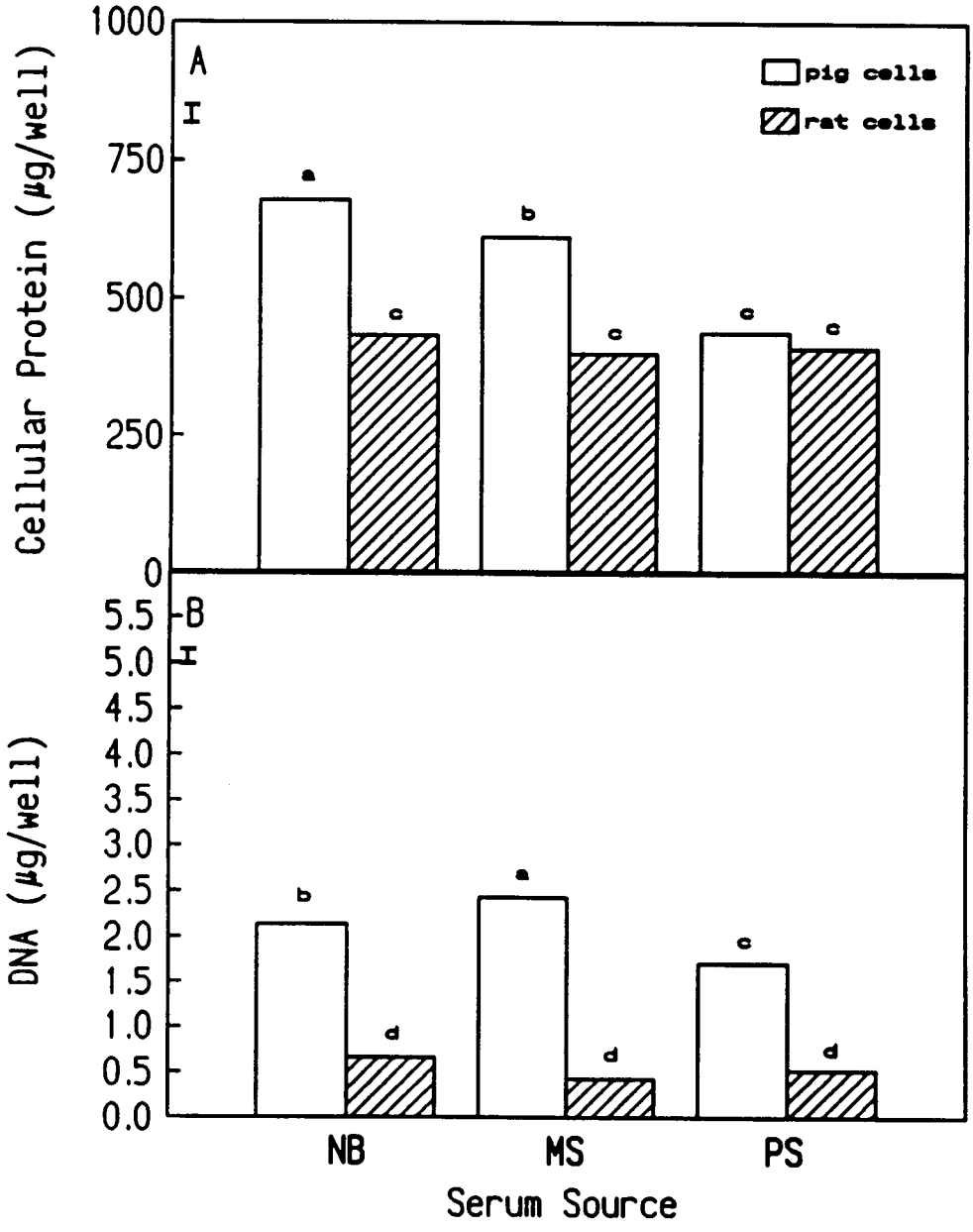
0.5
0.0
B
I
DNA (µg/well)
a
b
a
b
SR
FR
Serum Source

FIGURE 6.3

FIGURE 6.4. *Cellular protein and DNA contents of rat or pig adipose stromal-vascular cells cultured in serum from pigs of differing ages.* Cells were seeded on 6 well plates at a density of $3 \times 10^4$ cells/cm$^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% Sprague-Dawley rat (SR) or Fischer rat (FR) serum. Cellular protein and DNA contents in cultured cells were determined on d 12. Values are means of four independent experiments performed on triplicate wells. Bar represents pooled standard error. Means with the same letter are not significantly different at $P<.05$.


1000
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Cellular Protein (µg/well)
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b
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2.0
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0.5
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DNA (µg/well)
b
d
a
d
c
d
NB
MS
PS
Serum Source

FIGURE 6.4

FIGURE 6.5. *Cellular protein and DNA contents of cultured stromal-vascular cells obtained from inguinal or epididymal adipose tissue of male Sprague-Dawley rats.* Cells were seeded on 6 well plates at a density of $3 \times 10^4$ cells/cm$^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% Sprague-Dawley rat (SR) or Fischer rat (FR) serum or pig serum (PS). Cellular protein and DNA contents in cultured cells were determined on d 12. Values are means of two independent experiments performed on triplicate wells. Bar represents pooled standard error. Means with the same letter are not significantly different at $P < .05$.

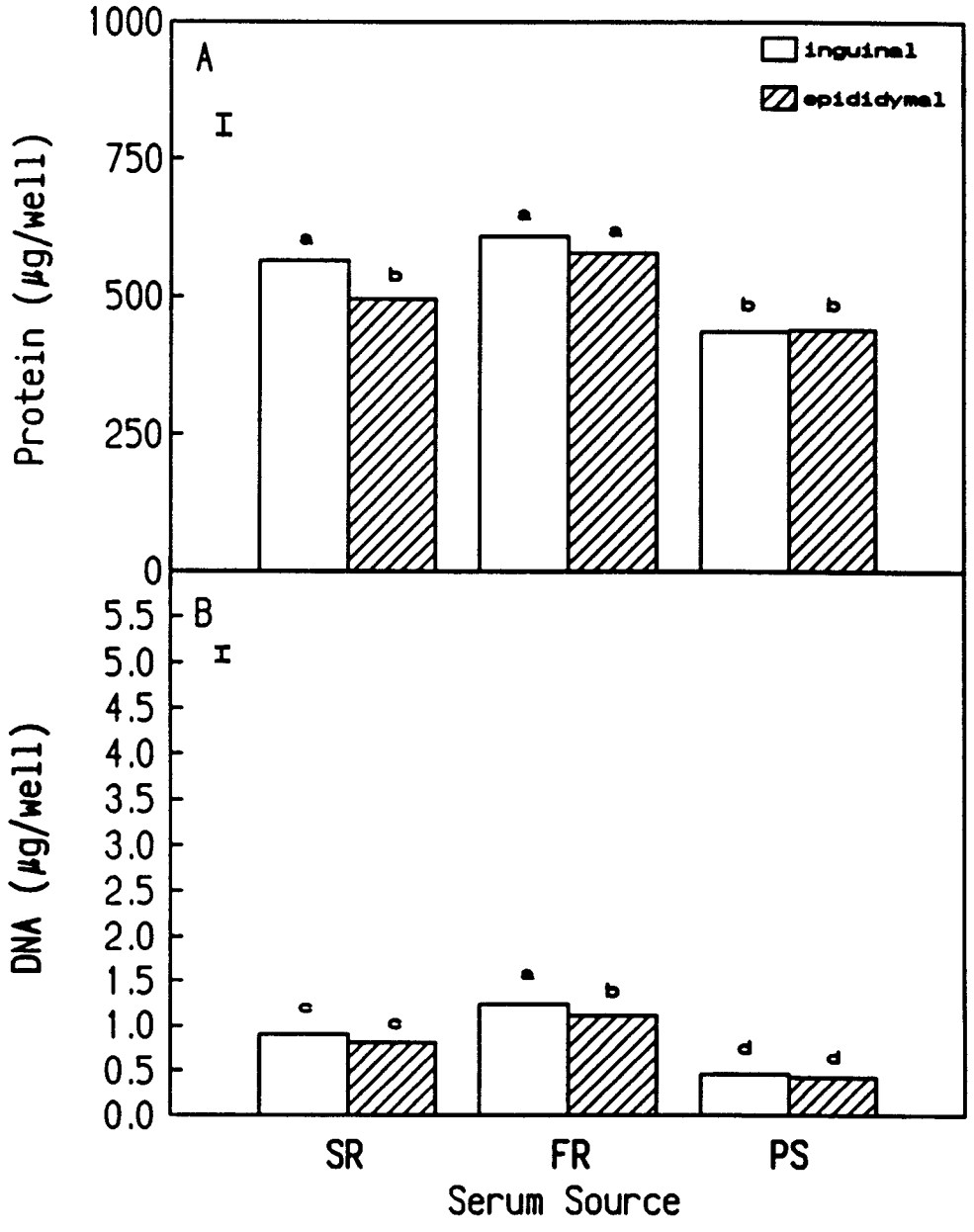
A
I
inguinal
epididymal
Protein (µg/well)
1000
750
500
250
0
a
b
a
a
b
b
B
H
DNA (µg/well)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
c
c
a
b
d
d
SR
FR
PS
Serum Source

FIGURE 6.5

FIGURE 6.6. *Sn-glycerol-3-phosphate dehydrogenase activity in stromal-vascular cells obtained from rat or pig adipose tissue.* Cells were inoculated on 6 well plates at a density of 3 x $10^4$ cells/$cm^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% Sprague-Dawley rat (SR) or Fischer rat (FR) serum . GPDH activity in cultured cells was determined on d 12. GPDH activity is expressed as specific activity (nmoles/min/mg protein; A) or GPDH per cell (nmoles/min /$\mu$g DNA; B). Values are means of four independent experiments performed on triplicate wells. Bar represents pooled standard error. Means with the same letter are not significantly different at $P<.05$.

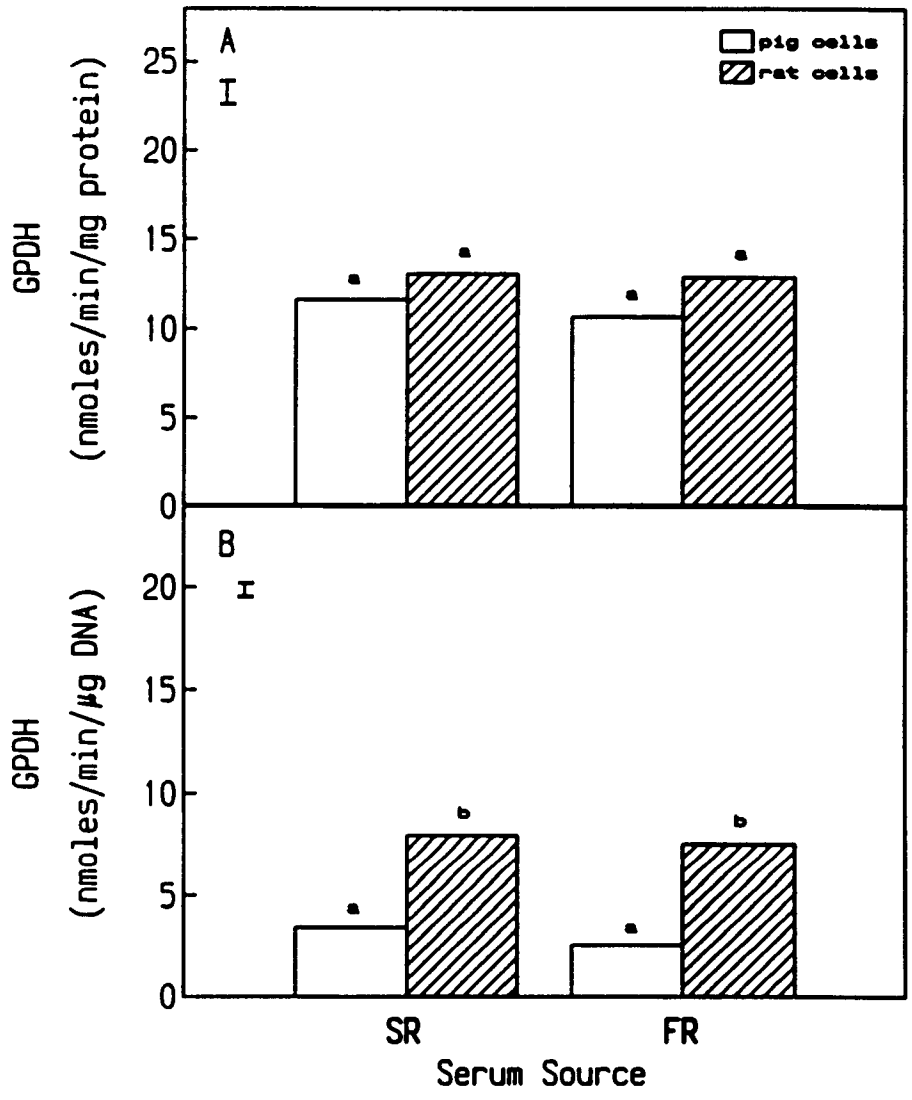
A
B
pig cells
rat cells
GPDH
(nmoles/min/mg protein)
GPDH
(nmoles/min/µg DNA)
0
5
10
15
20
25
a
b
SR
FR
Serum Source

FIGURE 6.6

FIGURE 6.7. *Sn-glycerol-3-phosphate dehydrogenase activity in rat or pig adipose stromal-vascular cells cultured in serum from pig of differing ages.* Cells were inoculated on 6 well plates at a density of 3 x $10^4$ cells/$cm^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% newborn (NB), 3 month old (MS) or 6 month old (PS) pig serum . GPDH activity in cultured cells was determined on d 12. GPDH activity is expressed as specific activity (nmoles/min/mg protein; A) or GPDH per cell (nmoles/min /$\mu$g DNA; B). Values are means of four independent experiments performed on triplicate wells. Bar represents pooled standard error. Means with the same letter are not significantly different at $P<.05$.

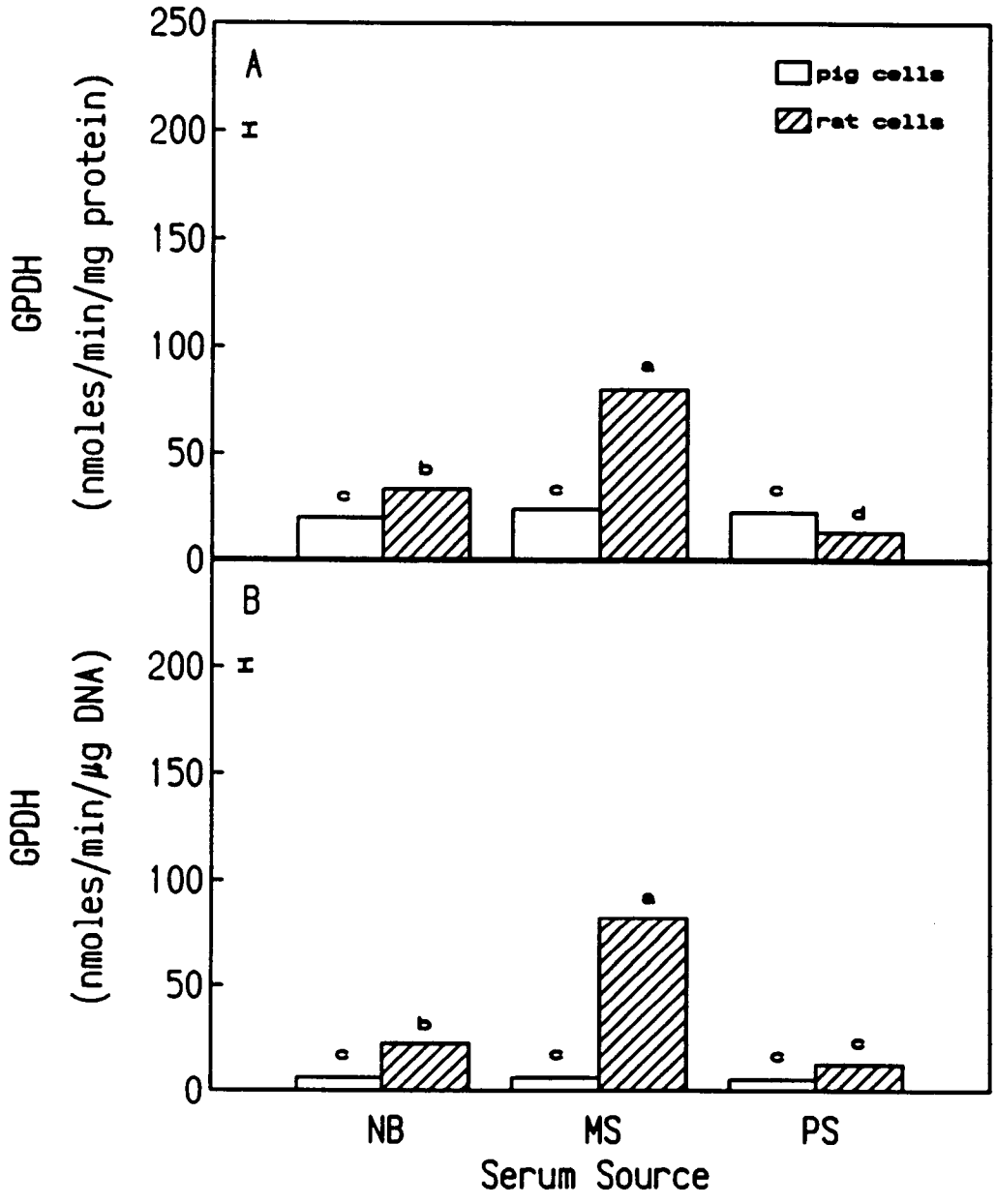
A
B
GPDH
(nmoles/min/mg protein)
GPDH
(nmoles/min/µg DNA)
250
200
150
100
50
0
pig cells
rat cells
a
b
c
d
NB
MS
PS
Serum Source

FIGURE 6.7

FIGURE 6.8. *Sn-glycerol-3-phosphate dehydrogenase activity in cultured stromal-vascular cells obtained from inguinal or epididymal adipose tissue of male Sprague-Dawley rats.* Cells were inoculated on 6 well plate at a density of $3 \times 10^4$ cells/cm$^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine supplemented with 2.5% Sprague-Dawley rat (SR) or Fischer rat (FR) serum or pig serum (PS). GPDH activity in cultured cells was determined on d 12. GPDH activity is expressed as specific activity (nmoles/min/mg protein). Values are means of two independent experiments performed on triplicate wells. Bar represents pooled standard error. Means with the same letter are not significantly different at $P<.05$.

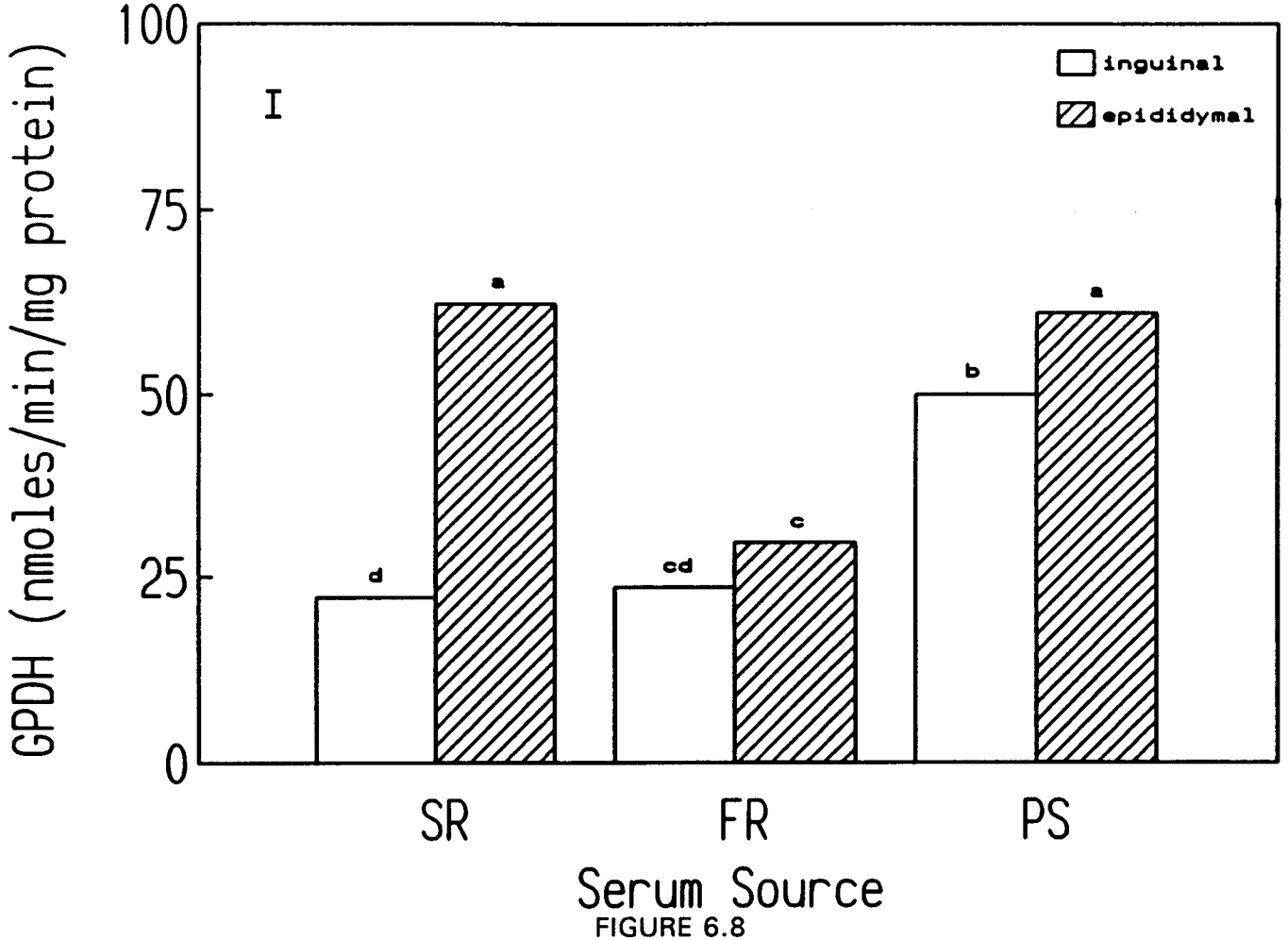
100
75
50
25
0
GPDH (nmoles/min/mg protein)
I
inguinal
epididymal
a
d
cd
c
b
a
SR
FR
PS
Serum Source

FIGURE 6.8

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# Chapter 7

# EFFECTS OF SERUM FRACTIONS ON THE DIFFERENTIATION OF CULTURED PORCINE ADIPOSE STROMAL-VASCULAR CELLS

## ABSTRACT

Fischer 344 rat and mature pig serum Sephacryl S-200 fractions were tested for their ability to promote porcine adipose stromal-vascular (S-V) cell differentiation. The elution profiles of the sera were similar. Rat serum contained an additional six protein peaks not present in pig serum based on absorbance at 280 nm, corresponding to molecular weight $\leq 2$ kD based on elution pattern of standard proteins. Rat serum fraction two (mol. wt. range 67-150 kD) stimulated GPDH specific activity and total GPDH in cultures of porcine adipose S-V cells as compared to cultures grown with whole rat serum and the corresponding pig fraction did not produce similar effect. Fraction three (mol. wt. range 17.8-42.7 kD) of both sera inhibited GPDH specific activity, total GPDH and lipid filling, in cultures of S-V cells but only rat fraction three promoted cell proliferation as compared to cultures grown in serum free medium. Other fractions showed variation in their ability to support or inhibit S-V cell differentiation. This study suggests that either concentration or compositional variations exist between pig and rat sera since fractions of each containing molecules with apparently similar sizes have different effects on the development of porcine preadipocytes. Further purification of each fraction of pig and rat serum will be required to determine composition and function in adipocyte development.

## INTRODUCTION

Blood is the medium through which circulating metabolites, hormones and growth factors are transported to individual cells. Growth and development of cells are influenced by extracellular environment. Sera from different species vary in their ability to promote differentiation and adipose conversion of both an adipocyte-like cell line (Kuri-Harcuch and Green, 1978) and rat preadipocyte primary cultures (Jewell and Hausman, 1989). Changes in serum borne components or factors significantly influence the ability of sera to regulate preadipocyte development (Ramsay et al., 1987).

Serum contains adipogenic factors necessary for adipose conversion and "anti-adipogenic" factors which are yet poorly defined or characterized (Loffler and Hauner, 1987). Some putative factors in bovine serum that inhibit the expression of adipocyte differentiation enzyme marker in Ob1771 cells have been identified (Pradines-Figueres et al., 1990). They are: low molecular weight lipophilic components (a serum lipid extract) and high molecular weight hydrophilic components (delipidated serum extract). Adipose conversion of 3T3 cells was influenced by an adipogenic factor in serum (Kuri-Harcuch and Green, 1978). Adipogenic factor in two species of genetically obese rodents (db/db mice and fa/fa Zucker rats) has been shown to be similar to that found in fetal calf serum (Loffler et al., 1983). A 63 kDa protein (Preadipocyte stimulating factor, PSF) was identified in rat serum; this protein may be highly species specific and may be

responsible for the promotion of greater adipose conversion and differentiation of adipocyte precursor cells by rat serum than any other sera (Li et al., 1989). There have been no reports on adipogenic or "anti-adipogenic" factors in pig serum.

The objective of this study was to evaluate the activities of the different rat and pig serum gel exclusion fractions on growth and development of porcine adipose stromal-vascular cells.

## MATERIALS

Dulbecco's Modified Eagle's Medium (DME, D-5523), Nutrient mixture F-12 (HAM, N-6760), dihydroxy acetone phosphate (DHAP, D-7137), reduced nicotinamide adenine dinucleotide (NADH, N-8219), gentamicin sulfate (G-1264), hydrocortisone (H-0135), insulin (I-1882), triiodothyronine (T-5516), bovine transferrin (T-8027), hematoxylin (HHS-2-16) were purchased from Sigma Chemical Co.(St. Louis, MO). Bovine serum albumin (BSA, Bovuminar Reagent CRG-7) was purchased from Armour Pharmaceutical Co. (Tarrytown,NY); collagenase (type I) from Worthington Biochemical (Freehold, NJ); fetal calf serum (FCS) from Intergen Co., (Purchase, N Y); Fungizone from Gibco BRL (Gaithersburg, MD); and Prepodyne from AMSCO, Medical Products Division (Erie, PA). Chromatography reagents, Sephacryl S-200 and molecular weight standards were purchased from Pharmacia LKB Biotech (Piscataway, NJ). All other reagents were of analytical grade.

## METHODS

### Animals and biopsy procedure

Crossbred pigs less than one day old from a commercial producer were killed by $CO_2$ asphyxiation. Pigs were scrubbed with Prepodyne and rinsed thoroughly with 70% ethanol solution, and placed on a sterile surgical tray in a laminar flow hood. An incision was made with a sterile scalpel through the skin from about .5 cm posterior to the base of the skull along the sagittal plane to the scapula and from the midline about 3 cm laterally on both ends. Adipose tissue in the exposed area was carefully removed from the underlying tissue with sterile forceps and scissors.

### Serum Sources

Serum from three month old male Fischer 344 rats was obtained from Hilltop Lab Animals, Inc. (Scottdale, PA). Blood was collected by aortic puncture at time of slaughter from crossbred barrows weighing 110-120 kg that have been maintained on ad libitum on corn soybean meal based diet. Blood samples were allowed to clot overnight at 4 °C. After clotting, the sera were separated by centrifugation at 800 x g for 20 min. Sera were pooled, filter sterilized using a 0.45 $\mu$m sterile acrodisc low protein binding filter (Gelman Sciences, Ann Arbor, MI), and aliquoted (3 ml) into 15 ml centrifuge tubes. Sera were stored at -20 °C until use.

### Gel Filtration Chromatography

Gel-exclusion chromatography of Fischer 344 rat and pig sera was performed at 4 °C using a 1.4 x 100 cm column of Sephacryl S-200 equilibrated and run in 0.005

M phosphate buffer, pH 7.5, containing 0.15 M NaCl and 0.04% (w/v) $NaN_3$. Three milliliters of Fischer 344 rat or pig serum were applied to the column. The flow rate was 10 ml/hr and the volume collected in each tube was 5 ml. Absorbance of each 5 ml fraction was measured at 280 nm on a Shimadzu UV 160U Spectrophotometer. The fractions collected were pooled by combining three successive 5 ml fractions together. The pooled fractions were dialyzed extensively in Spectrapor membrane tubing with molecular weight cut off of 6000-8000 (Spectrum Medical Industries, Inc.; Los Angeles, CA) against .005 M phosphate buffer, pH 7.5. The samples were lyophilized and dissolved in 3 ml DME/HAM's medium. The dissolved fractions were sterilized by filtering through 0.45 $\mu$m sterile Acrodisc low protein binding filter (Gelman Sciences, Ann Arbor, MI) prior to bioactivity tests. Unused fractions were stored at -20 °C.

Stromal-Vascular Cell Isolation

Dissected tissue samples were put in a petri dish containing Krebs-Ringer bicarbonate buffer (KRB, 37 °C; pH 7.4) containing 118 mM NaCl, 4.8 mM KCl, 10 mM HEPES, 5 mM glucose and 40 mg/L gentamicin sulfate, equilibrated with 95 % $O_2$:5% $CO_2$ and sterilized by filtering through a 0.22 $\mu$m filter (Nalgene Co, Rochester, NY). Adipose tissue samples (3 g) were minced with sterile scissors and digested for 1 h at 37 °C in a gyratory water bath in a 25 ml polypropylene flask with 9 ml filter sterilized KRB buffer containing 3 % BSA and 2 mg/ml collagenase. Digested tissue was filtered through a sterile single layer of polyester chiffon into 50 ml sterile polypropylene tubes. Floating adipocytes were separated from other

cells by aspiring the infranatant with a sterile syringe fitted with a long needle. The infranatant cell suspension was centrifuged at 800 x g for 10 min. S-V pellets were washed three times in DME/HAM's medium (1:1; v/v) containing 15 mM NaHCO3, 15 mM HEPES buffer (pH 7.4), 40 mg/L gentamicin sulfate and 2 mg/L Fungizone supplemented with 10% FCS (plating medium).

Cell Culture

Aliquots of the S-V cells were removed, stained with Rappaport's stain and counted on a hemocytometer. S-V cells were seeded in 3 ml plating medium on Corning 6 well (35 mm) tissue culture plates at a density of $3 \times 10^4$ cells/cm$^2$. Cells were cultured at 37 °C under a humidified atmosphere of 95% air: 5% $CO_2$; 24 hours later cells were washed 2 x 5 minutes and 1 x 1 hour with plating medium without FCS. Cells were subsequently maintained in test media. Test media consisted of plating without FCS supplemented with 20 $\mu$U/ml insulin, 1 ng/ml triiodothyronine, 25 ng/ml hydrocortisone, 10 $\mu$g/ml transferrin without supplementation (SF) or supplemented with 2.5% rat or pig serum (S) or serum fractions (2.5% serum equivalent). Test media were changed every 3 days until day 12 when cultures were terminated and protein, DNA and sn-glycerol-3-phosphate dehydrogenase were determined.

Enzyme Analysis

Sn-glycerol-3-phosphate dehydrogenase (GPDH; EC 1.1.1.8) was measured by a spectrophotometric method for determination of oxidized NADH during GPDH-

catalyzed reduction of DHAP (Kozak and Jensen, 1974) as modified by Wise and Green, (1979).

DNA and Protein Content

DNA was assayed as described by LaBarca and Paigen (1980) using salmon testes DNA as a standard. Protein content was determined by bicinchoninic acid method (BCA) using bovine serum albumin as a standard (Pierce Chemical Co., Rockford, IL).

Histochemistry

Representative wells from each treatment were fixed in 10% formalin, stained with oil red O for lipid and counterstained with Harris hematoxylin (Boon and Drijver, 1986) after 12 d of exposure to test media.

Statistical Analysis

Enzyme activity, DNA and cellular protein content data obtained from cultured cells were subjected to one-way analysis of variance using the general linear models procedure (GLM) and differences between mean were determined by Least Significant Difference (SAS, 1987).

## RESULTS

Figure 7.1 shows the elution profiles of 3 ml of serum from three month old male Fischer 344 rats and six month old pig serum pool from a 1.4 x 100 cm

Sephacryl S-200 column. Eighty 5 ml fractions were collected from each run of pig or rat serum. Protein determination based on absorbance at 280 nm of each 5 ml fraction indicated that protein eluted in rat serum fractions 12-47 and pig serum fractions 12-41. The elution profile was very consistent from one run to another and only slight differences were seen between absorbencies of peaks of corresponding fractions from both sera.

When three successive five milliliter fractions were pooled, twelve fractions from rat serum and ten from pig serum resulted. These fractions were eluted at 60-235 ml and 60-205 ml for rat and pig sera, respectively, as peaks corresponding to molecular weight range of $>150$ to $<1$ kD as calculated on comparison with standard proteins. Figures 7.2 and 7.3 show the effects of the pooled rat and pig serum fractions, respectively, on cellular protein and DNA contents of cultured porcine adipose S-V cells. Cellular protein and DNA contents were similar in cultures treated with fractions eluted at 130-235 and 130-205 ml (mol. wt. $\leq 8$ kD) for rat and pig sera, respectively, as compared to serum free (SF) medium after dialysis (data not shown). Cellular protein and DNA contents were highest in cultures treated with whole serum (Figs. 7.2 and 7.3). Cultures treated with rat fraction two contained significantly higher protein content compared to SF and other rat serum fractions while DNA content in cultures treated with rat serum fractions one, two and three were significantly higher than DNA content in cultures treated with SF (Fig. 7.2). Several of the pig serum fractions were not significantly different in their ability to affect cellular protein contents, but pig serum fraction two significantly increased cellular protein and DNA contents in cultured cells as

compared to SF (Fig. 7.3).

Figures 7.4 and 7.5 show the effect of the pooled rat and pig serum fractions, respectively, on GPDH activity, a marker enzyme of adipocyte differentiation in cultured S-V cells. Using GPDH activity of cells cultured in (SF) as an index, the results show that fractions eluted at 130-235 ml and 130-205 ml for rat and pig sera, respectively, promoted similar GPDH specific activity in cultured porcine adipose S-V cells as compared to SF after dialysis (data not shown). Supplementation of SF with 2.5% whole serum (S) decreased GPDH specific activity by 84 and 46 % for rat and pig sera, respectively. All pig and rat fractions tested decreased GPDH specific activity in cultured S-V cells as compared to SF. Rat serum fraction two (mol. wt. range 67-150 kD) promoted significantly higher GPDH specific activity compared with whole rat serum. Rat serum fraction two had quantitatively higher GPDH specific activity than other rat serum fractions but this was not statistically significant (Fig.7.4). Fraction three (mol. wt. range 17.8-42.7 kD) from both pig and rat sera produced effect similar to whole serum on GPDH specific activity. Pig serum fraction three significantly decreased GPDH specific activity in cultured S-V cells compared with either whole pig serum (S) or SF. Pig serum fraction three produced quatitatively lower GPDH specific activity than other pig serum fractions and the effect was significant ($P < .05$; Fig. 7.5)

When GPDH activity was expressed on per well basis rat serum fraction two (mol. wt. range 67-150 kD) produced quantitatively similar GPDH activity to SF; this activity was higher than all other rat serum fractions. All other rat serum fractions showed inhibitory effect. Cells cultured in rat serum fraction three had the lowest

GPDH activity per well (Fig. 7.4). Several of the pig serum fractions produced similar effct as SF when GPDH was expressed on per well basis but fraction three was inhibitory. S-V cells cultured in pig serum fraction three had the lowest GPDH activity per well which was significantly different from whole pig serum and other pig serum fractions (Fig. 7.5). Although elution profiles appeared similar only serum fractions three from pig and rat showed comparable activity. GPDH activity per unit DNA was highest in cells cultured in SF and lowest in whole pig or rat serum and fraction three of each (data not shown).

Figure 7.6 shows the photomicrograph of cultured cells. Fewer lipid containing cells occurred in cultures treated with pig serum fraction three compared to cultures treated with SF or whole pig serum, corresponding to GPDH measurement. Similar result was obtained with rat serum fraction three.

## DISCUSSION

The present study investigated the effects of pig and rat serum fractions separated on Sephacryl S-200 column. Comparison of the elution profiles of serum obtained from 3 month old male Fischer rats and 6 month old barrows indicates that the two sera are almost identical based on the number of protein peaks eluted. Rat has additional serum protein that eluted as low molecular weight ($< 2$ kD) serum components. Since corresponding fractions in pig serum are absent, this suggests that there are divergence in number of serum components from this two species. In addition there are variations in the levels of corresponding fractions from pig and rat since the absorbance of corresponding peaks at 280 nm vary. The variation

might be due to species differences or to certain physiological factors. Until specific factors are purified and physiological factors responsible for their production determined, what are responsible for the variations in levels of corresponding fractions from two different species could not be said with certainty.

The corresponding fractions from pig and rat were evaluated to determine their ability to promote growth and development of cultured S-V cells obtained from newborn pigs. Cells were also cultured in serum free and serum containing media to provide indices for which comparisons could be made. GPDH activity in cultured cells was used as a biochemical marker of adipocyte differentiation (Pairault and Green, 1979; Djian et al., 1983). DNA and protein were determined in homogenized cells as indicators of cellular growth and cell number. After dialysis of the serum fractions against phosphate buffer, GPDH activity in cells cultured with serum fractions eluted at peaks corresponding to molecular weight $<8$ kD were similar to GPDH in cells cultured with control media, SF, indicating that proteins or serum components in those fractions have been dialyzed out and the result obtained was from the control medium. Since the molecular weight cut-off of the dialysis membrane used was 6-8000 effect of serum components $<8$ kD on stromal-vascular growth and development could not be evaluated in the present study. A number of reports indicate that rat serum contains relatively small peptide with molecular weight of 4-6 kD having adipogenic activity that is certainly not insulin (Loffler et al., 1983). A peptide of molecular weight 6-8 kD purified from fetal calf serum has differentiating effect on ob17 cells (Grimaldi et al., 1982). It is not known if these small peptides are present in pig serum since the comparison of

elution profiles of pig and rat sera revealed that some serum components of low molecular weight are absent in pig serum. These small peptides may also not have a differentiating effect on porcine adipose stromal-vascular cells; it has been reported that the factors that promote differentiation and adipose conversion of cell lines are different from those that produce the same effect in primary culture (Li et al., 1989).

Serum contains adipogenic factors necessary for adipose conversion and poorly defined "anti-adipogenic" factors (Loffler and Hauner, 1987). Serum interferes with adipose conversion of cultured cells (Serrero and Mills, 1987) and serum free media promote a high rate of differentiation in stromal-vascular cells obtained from adipose tissue of rat and human (Deslex et al., 1986; Serrero and Mills, 1987) and rabbit (Reyne et al., 1989). The results of the present study are in agreement with the reports of Deslex et al.(1986), Serrero and Mills (1987) and Reyne et al. (1989). Addition of 2.5% pig or rat serum to SF caused reduction in differentiation activity in cultured cells (Figs. 7.4 and 7.53); however, some cells were still able to accumulate multilocular lipid droplets in their cytoplasm which were stained with oil red O on day 12 (Fig. 7.6). This indicates that some S-V cells grown in serum supplemented medium retained the ability to differentiate into adipocyte. Alternatively, these might be cells that are in the terminal phase of the differentiation process and ready for lipid filling. Serum promoted cellular growth and increased cell number as indicated by the cellular protein and DNA contents. Increased cellular growth and cell numbers might be attributable to increase in cell types other than preadipocytes. This conclusion is supported by the results of oil

red O staining. There are more numerous oil red O stained cells in cultures grown in SF as compared with SF supplemented with serum.

When the different serum fractions were evaluated for their differentiating ability, rat serum fraction two (mol. wt. range 67-150 kD) promoted similar differentiating activity as compared to SF when GPDH activity was expressed on per well basis (Fig. 7.2). Rat serum fraction one also promoted more cellular growth than whole serum or SF as indicated by cellular protein and DNA content in cultured cells. Activity of the corresponding fraction one from pig serum on cell differentiation differed completely from its counterpart from rat. This suggests that molecular weight might be the same but the nature of the active components of serum might differ from species to species. Preadipocyte Stimulating Factor (PSF) with molecular weight between 68 and 58 kD has recently been identified in rat serum. This factor is suggested to be highly species specific (Li et al., 1989). It is not known whether rat serum fraction two contains a component similar to PSF; further purification and characterization of this fraction is required. Fraction three from both pig and rat sera inhibited differentiation activity in cultured cells but rat serum fraction three was able to increase cell replication as compared to SF (Figs. 7.2 and 7.3). This shows that rat fraction three could play a dual effect on cell growth. Protein factor from rat adipose tissue that specifically stimulate proliferation of 3T3-L1 and Ob1771 cell lines and also inhibits preadipocyte differentiation has recently been identified. This protein has apparent molecular weight of 20 kD (Aoki et al., 1990). There are several other growth factors including platelet derived growth factor that fall into the molecular weight range of

fraction three obtained from Sephacryl S-200 column in the present study; further exploration is required to determine the nature of this inhibiting factor(s).

There are several growth factors in serum interplaying as positive and negative effectors. Identification of the different factors controlling adipocyte proliferation and differentiation will increase our understanding of adipose tissue development and possibly enhance our ability to define ways to control excessive fat deposition in the body.

FIGURE 7.1. *Elution profile of 3 ml of pig and rat serum from a 1.4 x 100 cm Sephacryl S-200 column.* The column was equilibrated with .005 M phosphate buffer, ph 7.5, containing .15 m NaCl and .04% (w/v) $NaN_3$. The flow rate was 10 ml/hr. Molecular weight markers were run and their relative elution position is represented by the arrows (catalase, mol wt 232 kilodaltons, aldolase, mol wt 150 kilodaltons, serum albumin, mol wt 67 kilodaltons, ovalbumin, mol wt 43 kilodaltons, chymotrypsinogen A, mol wt 25 kilodaltons and ribonuclease A, mol wt 13.7 kilodaltons). Five milliliter fractions were collected. Three successive fractions were pooled together to form individual fractions used for bioassay.

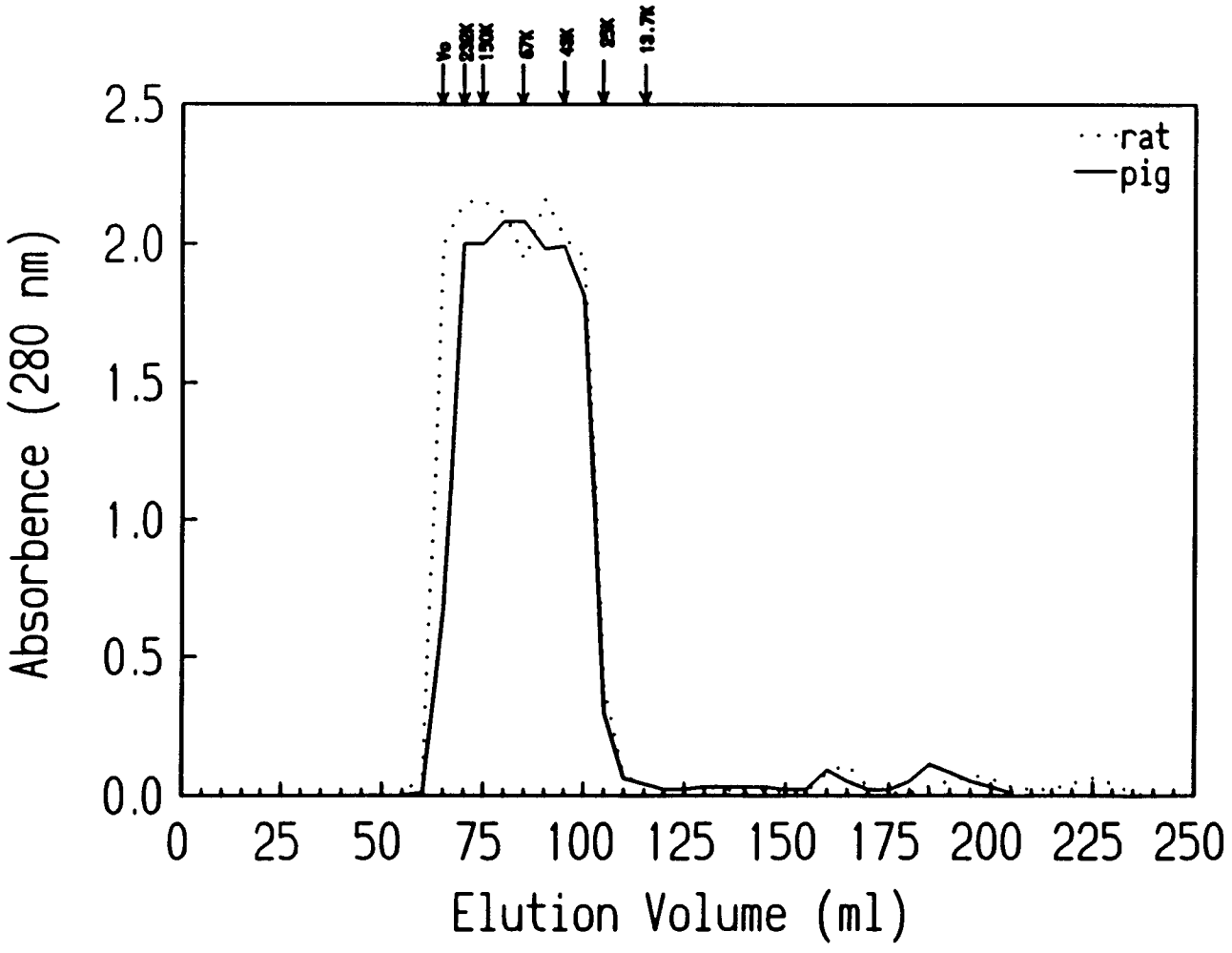
Vo
232K
150K
67K
45K
25K
13.7K
2.5
2.0
1.5
1.0
0.5
0.0
Absorbence (280 nm)
rat
pig
0
25
50
75
100
125
150
175
200
225
250
Elution Volume (ml)

Figure 7.1

FIGURE 7.2. *Protein and DNA contents of porcine stromal-vascular cells obtained from adipose tissue of newborn pigs cultured in rat serum or serum fractions.* Cells were seeded on 6 well plate at a density of 3 x $10^4$ cells/$cm^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's medium containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine (SF) supplemented with 2.5% whole rat serum (S) or rat serum fractions (2.5% serum equivalent). Protein (A) and DNA (B) contents in cultured cells were determined on d 12. Values are means ± SE of four independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P<.05$.

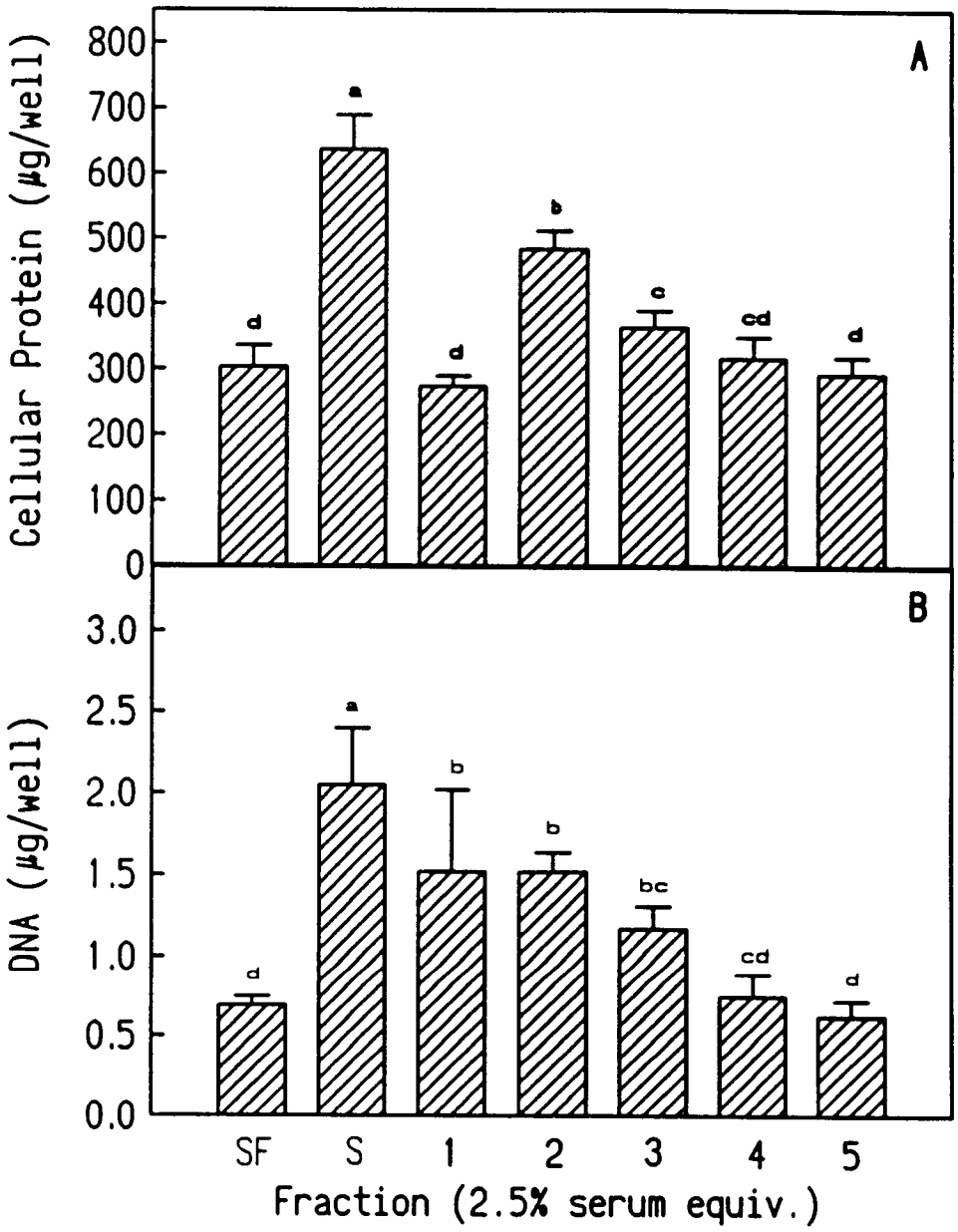
A
B
Cellular Protein (µg/well)
DNA (µg/well)
800
700
600
500
400
300
200
100
0
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
a
b
c
cd
d
a
b
b
bc
cd
d
SF
S
1
2
3
4
5
Fraction (2.5% serum equiv.)

Figure 7.2

FIGURE 7.3. *Protein and DNA contents of porcine stromal-vascular cells obtained from adipose tissue of newborn pigs cultured in pig serum or serum fractions.* Cells were seeded on 6 well plate at a density of 3 x $10^4$ cells/$cm^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's medium containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine (SF) supplemented with 2.5% whole pig serum (S) or pig serum fractions (2.5% serum equivalent). Protein (A) and DNA (B) contents in cultured cells were determined on d 12. Values are means ± SE of three independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P<.05$.

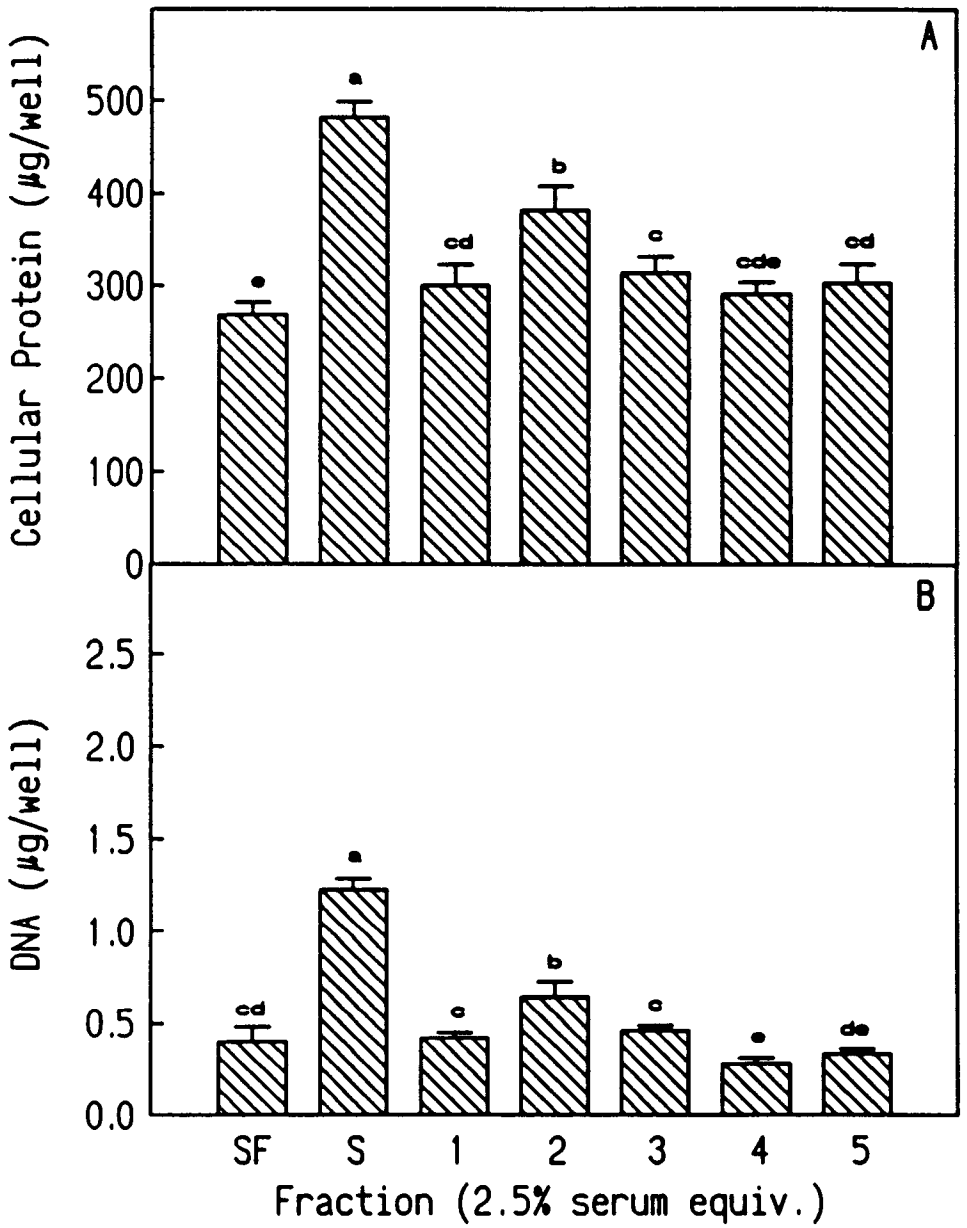
A
B
Cellular Protein (µg/well)
DNA (µg/well)
500
400
300
200
100
0
2.5
2.0
1.5
1.0
0.5
0.0
SF
S
1
2
3
4
5
Fraction (2.5% serum equiv.)

Figure 7.3

FIGURE 7.4. *Sn-glycerol-3-phosphate dehydrogenase activity in stromal-vascular cells obtained from adipose tissue of newborn pigs as influenced by whole rat serum or serum fractions.* Cells were inoculated on 6 well plate at a density of 3 x $10^4$ cells/$cm^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's medium containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine (SF), either supplemented with 2.5% whole rat serum (S) or rat serum fractions (2.5% serum equivalent). GPDH activity in cultured cells was determined on d 12. GPDH activity is expressed (A) as specific activity (nmoles/min/mg protein) and (B) as GPDH per culture well (nmoles/min/well). Values are means ± SE of four independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P < .05$.

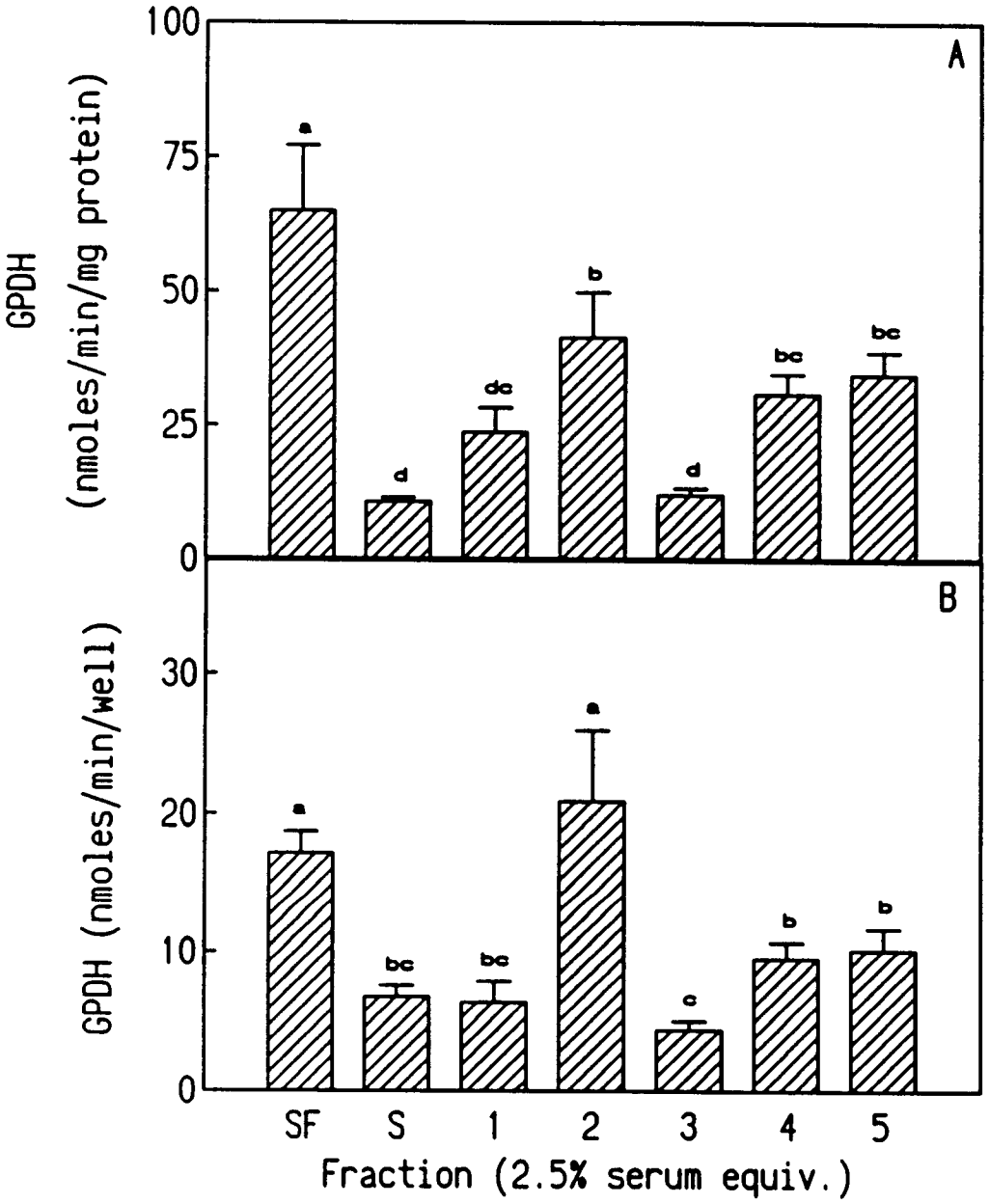
GPDH
(nmoles/min/mg protein)
100
75
50
25
0
A
a
d
dc
b
d
bc
bc
GPDH (nmoles/min/well)
30
20
10
0
B
a
bc
bc
a
c
b
b
SF
S
1
2
3
4
5
Fraction (2.5% serum equiv.)

Figure 7.4

FIGURE 7.5. *Sn-glycerol-3-phosphate dehydrogenase activity in stromal-vascular cells obtained from adipose tissue of newborn pigs as influenced by whole pig serum or serum fractions.* Cells were inoculated on 6 well plate at a density of 3 x $10^4$ cells/$cm^2$ in DME/HAM's medium supplemented with 10% FCS for 24 h. Cells were extensively washed in DME/HAM's medium without FCS and subsequently maintained in DME/HAM's containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine (SF), either supplemented with 2.5% whole pig serum (S) or pig serum fractions (2.5% serum equivalent). GPDH activity in cultured cells was determined on d 12. GPDH activity is expressed (A) as specific activity (nmoles/min/mg protein) and (B) as GPDH per culture well (nmoles/min/well). Values are means ± SE of three independent experiments performed on triplicate wells. Means with the same letter are not significantly different at $P<.05$.

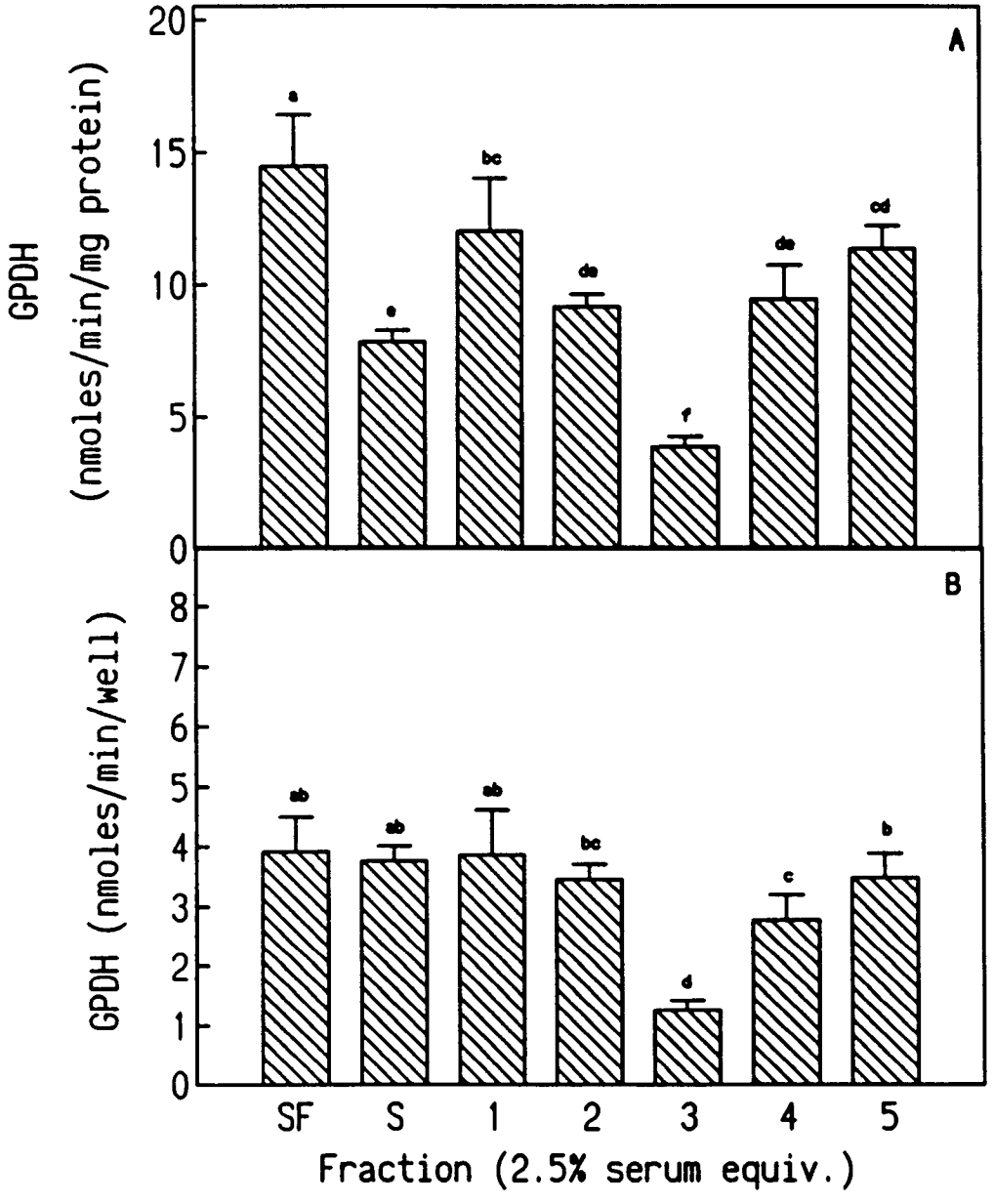
GPDH
(nmoles/min/mg protein)
20
15
10
5
0
A
B
GPDH (nmoles/min/well)
8
7
6
5
4
3
2
1
0
SF
S
1
2
3
4
5
Fraction (2.5% serum equiv.)

Figure7.5

Figure 7.6. *Photomicrographs of pig adipose stromal-vascular cells stained with oil red O and hematoxylin.* Cells were inoculated at a density of 3 x $10^4$ cells/$cm^2$ and grown in DME/HAM's medium containing 10% FCS for 24 h. after which cells were washed with DME/HAM's medium without FCS. Cells were subsequently grown in DME/HAM's medium containing 10 $\mu$g/ml transferrin, 20 $\mu$U/ml insulin, 25 ng/ml hydrocortisone and 1 ng/ml triiodothyronine (A) supplemented with (B) 2.5% whole pig serum or (C) pig serum fraction three (2.5% serum equivalent). On day 12 of culture, cells were washed with phosphate buffered saline, fixed in 10% formalin, stained with oil red O and counterstained with hematoxylin. Cytoplasmic lipid droplets were stained red with oil red O while nuclei were stained blue with hematoxylin. Note the fat cell clusters formed in cultures grown in whole pig serum and the inhibition of lipid deposition by pig serum fraction three. Magnification = 100x. Bar = 200 $\mu$.

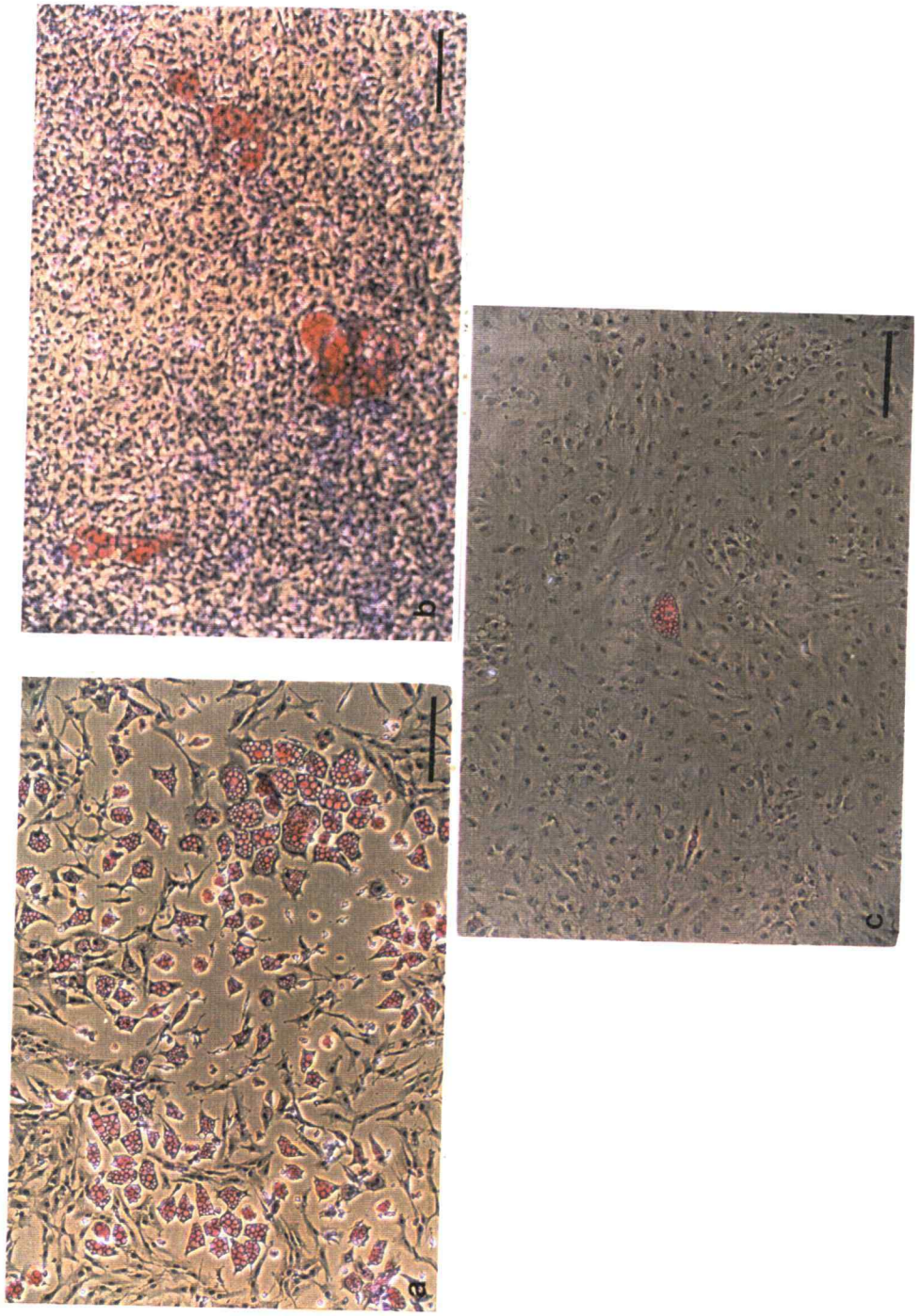


Figure 7.6

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## SUMMARY

Although the origin of adipocyte precursor cells has not been resolved, radioisotopic studies show that newly synthesized adipocytes come from primordial cells in the stromal fraction of adipose tissue. Culture of adipose stromal-vascular (S-V) cells under suitable conditions results in the development of cells with morphological and biochemical characteristics of mature adipocytes. The growth of S-V cells in culture provides us with a valuable model other than established cell lines, some of which have undergone transformation and are considered abnormal for the study of physiology and development of adipocytes.

Some studies of porcine adipose S-V cells in culture have used serum in the media because of the unavailability of suitable serum free systems. Serum contains many components of which some are poorly characterized or have not been identified, thus use of serum in cell culture medium has inherent disadvantage in the study of hormones and factors regulating adipocyte development. The presence in serum of specific growth factors and several undefined factors can confound the results of studies designed to evaluate the roles of these and other serum components that influence growth and development of adipocyte precursor cells. In the present study, S-V cells obtained from mature pigs were used to establish hormonal requirements of porcine adipose S-V cells in culture. Insulin and glucocorticoids are considered essential for the development of porcine S-V cells in culture. Insulin and glucocorticoids were included at physiological ranges with or

without triiodothyronine in a serum free system to study pig adipocyte development. Insulin, hydrocortisone, and triiodothyronine at physiological ranges were used in subsequent experiments and provided a suitable serum free medium for the culture of porcine S-V cells.

Several signals are responsible for triggering differentiation processes in cells. It is not known whether signals responsible for growth and development of porcine S-V cells come from cells themselves or are serum borne. The culture of S-V cells from newborn and mature pigs in sera from both ages reveals that intrinsic activities of the cells are responsible for the differences in growth and development of cells and not serum borne factors. Studies are needed to identify the differences between cells from newborn and mature pigs. This will provide target(s) wso that we employ cellular and molecular biology manipulation techniques to achieve body compositional changes in meat producing animals. The present study also revealed that serum from genetically lean pigs is more adipogenic than serum from obese pigs. It is likely that intrinsic activity of obese pig cells rather than serum borne factors is responsible for greater adipose tissue mass in genetically obese pigs. Genetically lean and obese pigs are good models for the study of extremes in tissue growth. Identification of cellular defects that are responsible for increased adipose tissue mass in obese pigs will increase our knowledge of adipocyte biology.

Fasted pig sera stimulated differentiation of rat and pig S-V cells and decreased proliferation of rat S-V cells as indicated by DNA data, suggesting that fasting can cause an increase in the levels and/or activities of factors influencing adipocyte differentiation or proliferation.

There are positive and negative growth factors in serum interplaying on growth and development of adipocytes. When sera from rat and pig were fractionated on a Sephacryl S-200 column, a serum fraction corresponding to a molecular weight range of 17.8-42.7 kD was isolated from rat and pig sera that inhibits pig S-V cell differentiation and lipid filling. Further purification and characterization of this factor is necessary so that its nature can be understood. The present findings show that rat and pig adipose S-V cells become morphologically different as they differentiate, and they appear to respond differently to the same stimuli. Caution should be taken when extrapolating from data from rat cell cultures to the situation in pigs.

The identification of various factors influencing adipocyte growth and development should prove beneficial in devising effective means of reducing body fat content in animals and should also make it likely for livestock producers to have bases for selection of animals with potential for leanness or for excessive body fat deposition.

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